A STUDY OF BARLOW'S DISEASE EXPERIMENTALLY PRODUCED IN FETAL AND NEW-BORN GUINEA PIGS.*

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Plate 38.

The relation between the organisms of the mother and offspring during the period of pregnancy has been the subject of thorough investigation. In both clinical and experimental pathology a number of studies, through a varied series of influences on the maternal organism, throws light upon the secondary influences on the fetal body. The agents employed to produce disease have usually been chemical, physical, or parasitic in character, but investigations as to the influence of dietetic changes upon pregnancy are limited in number. I may mention here particularly the dietetic experiments with strontium made by Lehnerdt[1] on pregnant rabbits. He succeeded in producing in young fetuses a disease of the osseous system, which exhibited macroscopically numerous fractures and infractions of the long tubular bones, and microscopically an increase in the osseous tissue, with extensive new formation of osteoid tissue and greatly impeded reabsorption.

In the present paper I wish to describe certain experiments made upon guinea pigs during pregnancy. The constant character of the frequently fatal disease that was produced in guinea pigs within a period varying from ten to twenty-eight days by a diet consisting exclusively of oats and water in Holst and Frölich's experiments[2] coincided closely both macroscopically and microscopically with Barlow's disease in man.[3] This, therefore, seemed to give an

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[1] Lehnerdt, F., *Beitr. z. path. Anat. u. z. allg. Path.*, 1909, xlvi, 468; 1910, xlvii, 215.

[2] Holst, A., and Frölich, F., *Ztschr. f. Hyg. u. Infectionskrankh.*, 1912, lxxii, 1.

[3] Ingier, A., *Frankfurter Ztschr. f. Path.*, 1913, xiv, 1.

opportunity of studying the possible changes in the osseous system of the fetus produced by a scorbutic diet, and also of obtaining some insight into the biological relation existing between mother and offspring. These studies might lead to conclusions with regard to the relation of the various constitutional osseous diseases of unknown etiology that occur spontaneously in man, the principal symptoms of which are dystrophy of the bone-forming elements.

The following diseases will be considered: osteogenesis imperfecta (congenital osteopsathyrosis), rachitis, and osteomalacia.

Before describing my experiments I wish particularly to emphasize the fact that the diet was tried on mature animals and on quite young ones, and also at various stages of pregnancy. The investigations showed that the stage of embryonic development as well as the resisting power of the pregnant animals is of importance, since in the experiments mainly older animals and those during the latter half of the period of pregnancy gave birth to living and apparently mature offspring, whereas experiments performed on young animals and those in the earlier stages of pregnancy invariably resulted in premature birth or in the birth of a dead fetus.

Thirty-three of my experiments were successful. They may be divided into three groups. Group I comprises the fetuses that were born early, prematurely or by miscarriage, within ten days after the beginning of the new diet. The second group comprises fetuses that were born prematurely or fetuses in an advanced stage of development between the tenth and twenty-fifth days. Group III comprises the young ones that were apparently fully developed and living, born within twenty-three days after the beginning of the experiment, which survived and were given a scorbutic diet; *i. e.*, milk from scorbutic mothers and later on oats and water.

The first group contains the litters of twelve animals. The fetuses had been alive for five to ten days after the experiment began. The litters consisted mainly of two, in some few cases of three or four animals. The young were either still-born or were found as dead fetuses in the uterus of the dead mother.

It appears that animals are more sensitive to changes in diet during pregnancy than at other times, since a comparatively large percentage of the mothers died in the early stages of the experiment.

All the fetuses showed a low stage of development. The weight varied from seventeen to fifty-five grams (the latter occurring once only), and the average was between twenty and thirty grams.

Macroscopically the bones presented nothing remarkable. Microscopic examination revealed no changes in the skeletons even of the longest living fetuses, with the exception of hemorrhage and thrombosis in the marrow.[4]

The second group comprises the litters of seventeen animals that had been fed during pregnancy on a scorbutic diet for twelve to twenty-five days. These cases present great variety. The degree of scorbutic change in the fetus was not proportional to the length of the period of intra-uterine dieting with scorbutic food. These changes, too, as regards intensity were considerably smaller than those in the skeleton of the mother. Thus, for instance, in one case after a diet of oats and water for twenty-two days, the offspring being alive and apparently fully developed (weight seventy-one grams), only isolated hemorrhages in the marrow and scarcely any perceptible reduction of osseous substance could be discerned under the microscope; whereas in another case, also after twenty-two days of experimental diet, the two young ones were born with pronounced Barlow's disease. They were still-born, undersized, apparently belonging to the first half of the period of pregnancy, although at the commencement of the experiment the stage of pregnancy, by palpation of the abdomen, seemed to be fairly advanced. It is, however, an extremely difficult matter to determine the exact stage of a litter.

Figures 1 and 2 show the microscopic structure of this case. They are microphotographs of one of the upper extremities. As in the other instances, there is no change in the cartilage. In the proliferative zone, the columnar zone, and the calcified layers of the cartilage, we find conditions that come within the scope of physiological variation. Destruction of cartilage has taken place almost symmetrically, by the formation of long columns of calcified ground substance that are partially connected into a network. These pillars sometimes continue subchondrally to the center of the bone. No

[4] As regards the purely technical aspect of the experiments, it may be stated that the bones were hardened in formol immediately after death, and subsequently partially decalcified in Müller's fluid. The method of staining employed was principally hematoxylin and eosin.

traces of osseous tissue can be found in the central or subchondral parts or in the zone of primary marrow spaces. Osteoblasts are also absent. The wide medullary spaces and the central medullary cavity are filled with structural marrow (*"Gerüstmark"*), consisting of fine fibers, spindle and stellate cells (medullary substance), in which only scattered specific osteomyelon cells are to be found.

The corticalis consists of a thin, osseous strip scarcely wider than a single row of osseous units where no apposition of osteoid tissue is discernible. In the center of the humerus a complete oblique fracture has taken place. The ends of the fracture have been displaced. The distal end has slipped along the outer surface of the upper one, and lies close to it, while the proximal end is wedged into the loose tissue of the cubital angle. At this spot copious and recent hemorrhage had taken place. We find here, moreover, numerous pigment cells. The periosteum, although torn, was not detached from the osseous tissue by the hemorrhage. At the seat of fracture there was hyaline degeneration of the periosteum and reaction was not discernible. At this point in the bone marrow connective tissue was beginning to be loosened.

In all the bones examined in this case abnormal length and persistency of the calcified columns of cartilage, deficiency of bone formation and medullary substance were found, and also fresh fractures and infractions in nearly all the long cylindrical bones. Even the scapula was fractured and only the ribs were whole. In all cases the hemorrhages were of small extent and few in number. Osteoclasts were very rare.

In reviewing these facts we must note that we are dealing with a case where the fetus corresponded to an earlier stage of development than could be expected from the time during which the pregnancy had been observed and the experimental dieting had lasted. The fetus, both macroscopically and microscopically, was backward in development.

On microscopical examination a perfectly normal appearance of the cartilage was revealed. The region of the endochondral ossification showed a deficiency of bone formation, since neither old nor recent osteoid tissue was discernible. The marrow was a typical medullary substance with few osteomyelon cells. As a rule, hemor-

rhages were rare. The periosteal formation was also diminished. Furthermore, if we take into consideration the fact that in numerous bones fractures and infractions of an undoubted intra-uterine origin were found, with merely a slight reaction from the periosteum and medulla, it is evident that we have all the symptoms of a fully developed case of Barlow's disease.

The second fetus of the case in question showed the same signs of the disease in an advanced stage. It is unnecessary to discuss the remaining cases of this group, as nearly all exhibited the disease to a lesser extent.

The litters comprised in group II were also premature. The weight was somewhat more than that given above; as a rule, between twenty-five and thirty-five grams. These figures were rarely exceeded. Thus in two cases in which birth occurred after twenty-one and twenty-two days respectively, the weights of the fetuses in one were sixty-four and sixty-eight grams, and in the other fifty-five and thirty-three grams. Of these litters only the latter exhibited marked scorbutic changes in the bones, in the form of medullary substance, sparse hemorrhage in the marrow, and markedly diminished osseous formation; whereas in the first litter, with the exception of loose teeth no changes were discernible in the skeleton, even with the aid of a microscope.

In the second group, too, the death of the mother took place comparatively often, five times out of seventeen, on the twelfth to fifteenth day of the experimental diet, and long before the expected time of delivery. The fetuses which were found in the uterus of the dead mothers all showed a stage of development approximately corresponding to the end of the first half of the period of pregnancy. Even after a lapse of twelve days these fetuses exhibited traces of a slight diminution of osseous formation. It is noteworthy that in one case a fetus showed advanced symptoms of Barlow's disease, exactly as described above, as early as the fifteenth day of experimental dieting; yet in this instance I observed the infraction of the corticalis in the subchondral zone, as is typical of Barlow's disease. The seat of infraction was the inner and upper end of the ulna, where it touches the head of the radius, and this fact may be of

importance as regards the origin of the fracture through intra-uterine movement.

Regarding the atypical seat of the fractures and infractions in these intra-uterine cases, they may perhaps be explained by assuming that the seat of fracture has moved towards the center by reason of the later growth of the bones. The pigment cells at the seat of fracture show that mainly older fractures are under consideration. The fact that in the case described an oblique fracture has taken place, agreeing entirely with one occasioned by indirect violence—by forcible bending—makes it probable, however, that the seat of the fracture is not dislocated by growth of the bone, but has taken place in the spot where it was found. The abundant fresh hemorrhage in this case also strengthens our argument. This difference in the seat of the fractures may be explained as follows: The predisposing factor of typical subchondral fractures in Barlow's disease may be sought in the circumstance that in this newly formed osseous zone a locus minoris resistentiæ is formed by the disease, which is, however, not the case with intra-uterine fractures, the corticalis being equally thin everywhere. It is noteworthy that there are no fractures of the ribs.

The origin of an intra-uterine fracture may be explained in this disease as well as in osteogenesis imperfecta as a mechanical action caused by contraction of the muscles (intra-uterine movements).

The results of my experiments prove beyond doubt that we can produce experimentally in the guinea pig embryo during its fetal existence a disease characterized by all the main features of true Barlow's disease.

In the third group of my experiments, comprising four cases from a later stage of pregnancy, the fetal skeleton, having reached a further development, displays a more marked picture of the disease, which resembles the scurvy of the adult animal still more. The secondary changes, however, are not so pronounced as those found in adult guinea pigs that were fed on oats and water until death.

The cases comprised in group III are all remarkable for the high degree of development of the fetus. They all appear to be fully developed and alive at birth. The weight was fifty-five to sixty-five grams.

The duration of experimental dieting, intra-uterine and extra-uterine, was as follows:

Case 1, 23 dys. intra-uterine, 5 dys. extra-uterine (mother's milk, oats, and water).
Case 2, 13 dys. intra-uterine, 16 dys. extra-uterine (mother's milk, oats, and water).
Case 3, 8 dys. intra-uterine, 7 dys. extra-uterine (mother's milk, oats, and water).
Case 4, 14 dys. intra-uterine, 5 dys. extra-uterine (mother's milk, oats, and water).

The most remarkable feature of these cases was the slight development of the secondary changes in the zone of ossification, which is so marked in human cases of Barlow's disease. This results from the slighter hemorrhage in the bone marrow, and in consequence the lesser disturbances of the circulation in the zones of subchondral and primary marrow spaces. In these cases, too, even after sixteen days of extra-uterine life, there results a symmetrical destruction of cartilage, with no changes in the cartilage, even in the bones where usually we find the highest degree of change; *viz.*, the ribs and extremities of the knee joints. The newly formed columns of calcified primary cartilagenous substance are more scarce than in normal animals; they are usually short and thick; only a few continue subchondrally as more substantial osseous columns. The subchondral spongiosa consists of scattered, isolated, somewhat small calcareous columns, on which only small quantities of osseous substance are deposited. Neither here nor in the zone of primary marrow spaces are there any traces of osteoid tissue. Osteoblasts are also rare, and exclusively spindle-like in shape. The corticalis is very thin, especially in the subchondral zone.

In nearly all cases we can trace subchondral infractions of the corticalis, and also isolated broken cartilage columns. These fractures are most pronounced in the offspring of the second case, which were influenced by the injurious diet for thirteen days *intra uterum,* and sixteen days *extra uterum,* and lived longest of all the animals experimented upon.

In the ribs, and particularly in the tibiæ, there were subchondral fractures with extensive, fresh hemorrhages in the periosteum and bone marrow. The circumstance that the epiphysial centers of ossification of the bones contained extensive fresh hemorrhages in the marrow proves that the latter were not only of traumatic nature, but

also were due to a hemorrhagic diathesis. Also the characteristic formation of medullary substance in the subchondral zone in all cases leads us to the conclusion that in these cases the changes in the skeleton increase not only with the duration of experimental feeding, but first and foremost with the mechanical strain on the skeleton of the extra-uterine animal. This seems to be directly confirmed by the circumstance that infractions and extensive hemorrhages were lacking in one of the offspring of case 1 of this group, which was killed immediately after birth, after twenty-three days of intra-uterine life, while diminished formation of bone and formation of medullary substance were discernible.

DISCUSSION.

From the experiments here described it is evident that a disease of the skeleton may be produced not only in the pregnant guinea pig, but also in the fetus, by feeding the mother exclusively on oats and water. The condition thus produced is characterized as Barlow's disease by the diminished or even non-existing formation of bones, by the existence of hemorrhages in the bone marrow, by the formation of medullary substance, and by the occurrence of fractures.

During the first half of the period of pregnancy the lack of the conditions necessary to sustain life, occasioned by the special diet, is so marked that the animals die almost at the beginning of the period of experiment, and in these cases microscopic examination of the skeleton shows only hemorrhages and thrombosis in the bone marrow.

In group II of my experiments I succeeded in keeping the animals alive for a somewhat longer period. In these cases, too, the fetuses were undeveloped, apparently belonging to the first half of the period of pregnancy, and were frequently still-born. In these cases I found, besides hemorrhages in the marrow, a more or less marked reduction of bone formation, in some instances complete cessation, a development of typical medullary substance and even undoubted intra-uterine fractures.

More marked secondary changes of the bones occurred after birth only by the consequent mechanical strain on the skeleton, the animals

being deprived continuously of the necessary materials for normal development of the bones, by dieting them on milk from a scorbutic mother, and on oats and water only. These secondary changes consisted in larger fractures in the epiphysial zone, indications of a more or less pronounced zone of decay, more extensive hemorrhages in the periosteum and bone marrow, and a reaction of connective tissue by the osteomyelon, corresponding exactly with a fully developed case of Barlow's disease as seen in adult animals after twenty-eight days of an oat diet. No case, however, showed a more marked disturbance of the endochondral reduction, or change in the cartilage proper.

On comparing the cases of Barlow's disease in the mother animal and in the offspring, it is seen that the life of the fetus is not of longer duration than the life of the mother, and this is true in cases fed with scorbutic food during the intra-uterine life, as well as in cases in which this period is succeeded by a period of postnatal scorbutic feeding. None of the fetuses were alive for more than twenty-eight days; that is, they have the same duration of life as the adult animals under the same conditions. On this point, mothers and young animals give the same results.

On the other hand, both macroscopic and microscopic changes in the skeletons of the fetus, although pronounced, were evidently less than those in the adult animal. Thus the zone of decay was always lacking and, accordingly, the secondary changes in the cartilage. Therefore the offspring that were born alive were lively and active. Several points must be considered in order to explain this circumstance. I am inclined to believe that the first cause is the vital independence of the fetus in the uterus. In the case of diseases occurring spontaneously, as well as in injury occasioned by experiment to pregnant individuals, the damage to the organism of the fetus is always less than to that of the mother. We know that the state of nourishment of the mother does not in the least correspond in normal pregnancy with that of the new-born child. On the other hand, Goldmann's[5] experiment with vital staining proves directly that during the development of the fetus there is established a "kind of

[5] Cited by Wolff, B., in von Meyer and Schwalbe, Studien zur Pathologie der Entwicklung, Jena, 1913, i, 50.

center," to which "certain nutritious substances, *e. g.,* glycogen and fat, are directly attracted from the maternal organism."

Moreover, remembering that pregnant animals show a more advanced state of the disease in their bones at an earlier period of defective diet than is the case with non-pregnant animals, it appears that by a reduction of the amount of the essential components of diet, the fetus absorbs a comparatively high percentage of those substances, by reason of its superior intensity of assimilation.

Another important cause of the lesser development of osseous changes in the fetus is no doubt to be found in the circumstance that its protected position in the uterus wards off all but a few minor mechanical influences. This is shown again by the circumstance that very few days of extra-uterine existence are sufficient to produce marked symptoms of Barlow's disease even in cases where at birth the disease occurs only in a latent state. I was able to trace only slight disturbances of the skeleton in the case of young animals of the third group that were killed immediately after birth.

In these experiments we notice the high percentage of premature births and of still-born litters. I do not know whether any corresponding phenomenon has been observed in human beings during scorbutic epidemics, nor are there any accounts of cases of congenital Barlow's disease in the medical literature; and considering the long period of development of this disease in human beings and the rapid recovery caused by suitable diet, this circumstance is not remarkable. Judging from the complete correspondence of the disease in human beings and in guinea pigs, we must assume that such cases may exist. On the other hand, Cheadle and Poynton[6] mention scorbutic offspring whose mother suffered from the same disease.

With reference to the etiology of Barlow's disease when produced *intra uterum,* there is no doubt that unsuitable diet occasions a species of pathological assimilation that equally influences the organism of both mother and offspring. We may presume that a certain substance is lacking in the food, which substance is equally essential to both mother and infant. Yet even these experiments disclose

[6] Cheadle, W. B., and Poynton, F. P., in Allbut, C., and Rolleston, H. D., A System of Medicine, 1909, v, 898.

nothing as to the real nature of this action, whether directly on the bone-forming elements of the osteomyelon or by way of some inner gland secretion, or perhaps by a species of intoxication. It has occasionally been shown that by an insufficiency in the mother of a gland with inner secretion, a vicarious hypertrophy of the same gland occurs in the fetus. Thus the thyroid gland in the fetus is hypertrophied when that gland is removed from the mother (Halsted and others).

We may imagine something of the same kind in the case before us, but I have found nothing macroscopically that would lead to such an interpretation. No microscopic examination, however, has been undertaken with this in view. Undoubtedly the fetus cannot remain alive for a longer period than the adult animal, nor can it defend itself against the development of the disease.

At the same time, experiments made for the purpose of determining a possible failure of some secretive gland in adult animals gave no positive results. By the addition of various preparations of glands influencing the growth of bones (thyroid, hypophysis, thymus, parathyroid) to the oats and water diet, no positive results were obtained in a single case, either *per os* or parenterally. Nor did I succeed in influencing the disease by parenteral introduction of defibrinated blood from normally fed guinea pigs, in which presumably secreted substances from all the glands are present.

On the other hand, no experimenter has so far succeeded in a direct demonstration of the possible lacking substance in scorbutic diet. In this connection it is interesting to recall Schmorl's[7] investigations on Heubner's dogs, in which by a diet deficient in phosphates he found the symptoms of a disease that showed great likeness to Barlow's disease, but which could not be identified with it. Experiments which I have performed on adult guinea pigs point in the same direction, inasmuch as I did not succeed in checking the development of the scorbutic phenomena by adding phosphorated cod liver oil to the oats diet.

As regards the relation between the experimental results given here and the congenital disease osteogenesis imperfecta (congenital osteopsathyrosis) which occurs spontaneously in human beings, and

[7] Schmorl, G., *Arch. f. exper. Path. u. Pharmakol.*, 1913, lxxiii, 313.

which also is due to a diminution of the osseous development, it is clear even from the morphological and symptomatological discoveries in both instances that they differ essentially. The fact that I did not succeed in producing a disease resembling it, even by an alteration in the experiments whereby the injurious influences were less extensive, although allowed to continue for a longer period, is in accord with the above hypothesis. The circumstance is in full accord with the demands for a specific etiology of Barlow's disease, whereas the etiology of osteogenesis imperfecta is still unknown, whether occasioned by an injury to the ovum, or to the embryo at a very early stage.

By adding thirty grams of cabbage, boiled for one-half hour at a temperature of about 110° to 120° C., to the daily ration of oats, Holst and Frölich succeeded in keeping the animals alive for several months; when death occurred as the result of this diet, the animals showed scorbutic changes. As mentioned above, I failed to produce during this modification of the experiments an injury to the bone-forming elements coincident with their formation, which would assert itself in the later development of the skeleton. This diet also resulted in premature delivery; the fetus showed no particular changes in the skeleton; only lesions of scorbutic origin were observed.

These experiments, however, are of special interest on account of another disease of the bones which occurs in human beings during pregnancy; *viz.*, osteomalacia. Although exacerbation always occurs during pregnancy in this disease, I am not aware of any case in which a congenital osteomalacia or rachitic disease has been traceable in the fetus or in full term children of such mothers.

With respect to the total of the examples of congenital rachitis recorded in medical literature, even after eliminating osteogenesis imperfecta and chondrodystrophia fetalis, which were formerly regarded as fetal rachitis, the remainder consists essentially of cases based upon macroscopic observations. On the other hand, no authors, not even Schmorl in the course of his extensive examinations of the skeletons of human fetuses and the newly born, have so far succeeded in finding the pathognomonic aspect of the microscopic diagnosis of rachitis,—excessive periosteal superposition or thickening

and plane extension of the osteoid seams. Nor can the macroscopic appearance of intumescences at the limits of the cartilage be considered pathognomonic for rachitis, because the former exist as secondary symptoms in several osseous diseases; *e. g.,* rachitis, Barlow's disease, traumatic disturbances, and recently the "snuffles" in hogs.[8]

The circumstance that no child suffering from rachitis or osteomalacia has been known to be born of a mother suffering from osteomalacia during pregnancy, even when the disease has lasted throughout the full period of gestation, assuming that this factor will assert itself in future investigations, indicates that the two diseases are fundamentally different both as to their nature and their etiology. It is all the more noteworthy because Hart[9] claims to have discovered a case of rachitis among his cases of Barlow's disease during a series of experiments on monkeys, all of which had been put on the same scorbutic diet, and lived under the same conditions. He assumes a common etiology between Barlow's disease and rachitis.

We are thus concerned, on the one hand, as regards osteogenesis imperfecta with a disease in which an injury to the fetus may occur without any traceable illness in the mother, which seems to indicate a primary injury to the fetus. On the other hand, in osteomalacia we observe that a healthy fetus may be born of a constitutionally diseased mother. By the selective action of the placenta, that is, of the chorionepithelioma, the fetus is able, in the latter disease, to avoid the influence of the factors that are injurious to the mother.

While in the case of Barlow's disease the assumption, as an etiological factor, of a deficiency of some nutritive substance essential to the support of life as well as to the action of the bone-forming cells of the osteomyelon, would appear to be probable, we might perhaps in osteomalacia imagine a hormonic effect. The general relation between various secretive glands and the growth of the bones is well known. Thus, for instance, the disturbance of the function of the hypophysis is connected in some cases with acromegaly (Fischer and others), and in the same way the parathyroid glands are probably closely connected with the disturbances of osteomalacia (Erdheim, Todyo).

[8] Ingier, *Frankfurter Ztschr. f. Path.,* 1913, xii, 270.

[9] Hart, C., and Lessing, O., Der Skorbut der kleinen Kinder, Stuttgart, 1913.

CONCLUSIONS.

1. Pronounced cases of Barlow's disease may be produced in the fetus as early as ten to fifteen days after the commencement of dieting pregnant guinea pigs with oats and water. There are wide individual variations. The scorbutic changes in the skeleton are greatest in the earlier embryonic stages. The fetuses of that period, with practically no exceptions, die and show marked traces of impeded growth.

2. Fetuses from the later period of pregnancy are born alive, and apparently fully developed, with comparatively slight changes in the osseous system.

3. Even a short extension of the period of extra-uterine dieting on milk from scorbutic mothers and later on oats and water is sufficient to change the latent scurvy into a highly pronounced case.

4. The fetus cannot be kept alive longer than the adult animal, about twenty-eight days, either by intra-uterine dieting alone or by combined intra- and extra-uterine dieting.

5. The mothers show signs of disease at an early period, and are more severely attacked than non-pregnant animals. Death also occurs comparatively often in the first period of gestation.

EXPLANATION OF PLATE 38.

FIG. 1. General view of the humerus, ulna, and radius. At the limits of the ossifying cartilage in the humerus both metaphyses show a close network of calcified primary cartilagenous substance, with normal conditions in the cartilage; there is no endochondral formation of bone. The corticalis is thin throughout. In the middle of the bone there is a dislocated oblique fracture. The proximal piece is wedged into the loose tissues at the angle of the elbow and surrounded by hemorrhages. The periosteum shows hyaline degeneration at the seat of fracture, but no formation of callus. The marrow, which is typical structural medulla, contains a slight reaction of tissue at the fracture.

FIG. 2. The proximal extremities of the ulna and the radius, more highly magnified. Normal conditions in cartilage. (The black spot is due to a fault in preparation.) At the limits of ossifying cartilage, especially in the radius, is seen a close network of calcified primary cartilagenous matter, which continues in long calcified cartilagenous columns. In other sections the latter extend almost to the center of the bone. No endochondral formation of osseous matter is present. There is typical medullary substance throughout the bone. The corticalis is evenly thin, and the ulna shows in the middle of the bone an infraction of the corticalis with a slight thickening of the cambium layer at that point.

FIG. 1.

FIG. 2.

(Ingier: Barlow's Disease Experimentally Produced.)

PURE CULTIVATION IN VIVO OF VACCINE VIRUS FREE FROM BACTERIA.*

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PLATES 39 TO 50.

In spite of much effort no method has, up to the present time, been perfected by which vaccine virus can be propagated in a pure state, free from contaminating bacteria. The method of propagating vaccine virus universally practiced today consists in transmitting the virus from the skin of one calf to that of another. Although the inoculation of the virus is carried out under precautions as strictly aseptic as possible, the fresh product nevertheless yielded by the skin contains a not inconsiderable number of different bacteria derived from the skin surfaces, the air, etc.

The employment of glycerin as an elective germicide against the non-spore-bearing bacteria contained in fresh, or so called "green," vaccine pulp results in a great reduction both in number and variety of the contaminating microörganisms, without at the same time seriously impairing the activity of the vaccine virus (1). After contact with concentrated glycerin, in a refrigerator, ranging from one to three months, the virus usually is freed from most of the bacteria and becomes "ripe" for practical use in vaccination on human beings.

Different samples of the ripe vaccine preparations, as issued from various authorized sources, vary in their activities as well as in their germ contents. Among the organisms which may be encountered in such preparations are the following: streptococci, staphylococci, *Bacillus coli, Bacillus welchii, Bacillus xerosis, Bacillus subtilis,* and some other aerobic and anaerobic bacteria (2). The action of glycerin on bacterial spores, which may also be present in the pulp, is

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almost nil. Moreover, the prolonged action of glycerin tends gradually to reduce the activity of the vaccine virus itself, so that the fresh vaccine pulp forfeits some of its efficacy while undergoing the "ripening" process. At the end of the ripening process, which consumes from one to three months, the virus usually still contains a certain number of bacteria; bacteria-free virus is practically never produced by glycerination. But the residue of living bacteria, fortunately, is to all intents and purposes quite harmless and practically to be disregarded. And yet it is apparent that it is not only desirable to eliminate all bacteria from vaccine virus, but the saving of the time lost in "ripening" and the preservation unimpaired by glycerin of the full strength of the virus are also highly important considerations.

HISTORICAL.

The observations of Béraud (3), Weigert (4), Chiari (5, 6), Councilman, Magrath, and Brinkerhoff (7), and others (8) indicated that the variola virus localizes in different viscera and organs; the vaccine virus, on the other hand, according to Calmette and Guérin (9), and von Prowazek and Yamamoto (10), exhibits a high degree of affinity for pavement epithelium. When the vaccine virus is introduced directly into the blood or peritoneal cavity, it lodges in the epithelia of the skin and mucous surfaces, provided they are injured within a few hours of the inoculation, while in a few hours more it has completely disappeared from the circulation or the peritoneum. Should the epithelium not take it up, it is entirely lost.

In order to test the question whether still other epithelial cells take up the virus, Henseval and Convent (11) conducted in 1910 a series of experiments on the testes of rabbits. The virus that they employed contained staphylococci and *Bacillus subtilis*.[1] The effect was to produce some swelling, but no induration of the organ. Five to seven days after inoculation the testes were extirpated, emulsified in 66 per cent. glycerin, and tested on the shaved skin of rabbits. The activity was less than that of the original material. From the first testicular emulsion a second set of rabbits was injected; the swelling and induration produced was greater, but the activity of the emulsion was no stronger. No attempt was made either to free the vaccine of bacteria or to continue the testicular passages. Later, Paschen (12) injected vaccine (its bacterial content is not stated) into the testicle of a rabbit and noted the production of necrosis and cellular infiltration. As early as 1904 Tyzzer (13) attempted to obtain characteristic vaccinia lesions by inoculation of the testis of rabbits and a calf; the result was regarded as an acute inflammatory reaction. Von Prowazek and Miyaji (14) state that vaccine virus injected into the testicle of the rabbit can

[1] The number of contaminating bacteria in the three samples employed was 4, 10, and 25,000 per 0.01 c.c., respectively.

still be detected two days later, and that local necrosis of the tissue appears at the inoculation site. Nothing is said of the bacterial content.

That the testicle of the rabbit affords a favorable site for the multiplication of parasitic microörganisms can be inferred from the experiments of Parodi (15) and later of Uhlenhuth and Mulzer (16) with *Treponema pallidum*, of Nichols (17) with *Treponema pertenue*, and of a host of later workers with *Treponema pallidum* (18). I therefore turned my attention to the cultivation of pure strains of vaccine virus in the rabbit's testicle.

PURIFICATION OF VACCINE VIRUS PRIOR TO CULTIVATION.

At the outset and before describing the details of the experiments on the cultivation of vaccine virus it is necessary to point out a material difference between the employment of the rabbit's testicle for *Treponema pallidum* and for vaccine virus. In the case of the former the multiplication of the spirochætæ takes place slowly, so that the contaminating bacteria which are carried into the testicle with the inoculated fragments of syphilitic tissue may in time be suppressed by the bactericidal action of the vigorous tissues. On that account pure strains of the pallidum may be thus developed after a few passages. Vaccine virus, on the other hand, multiplies quickly, and the inflammatory reaction and necrosis of tissue which ensue quickly suppress the bactericidal process of the testicular tissues and place them in a favorable state for bacterial development. On that account the virus must first be freed from the bacterial contamination by other means.

Preliminary Purification.—Several antiseptic or disinfecting substances may be employed for reducing or removing contaminating bacteria. Glycerin is universally employed in practice to reduce the number, because of the small effect which it exerts on the virus itself. At low temperatures the antiseptic action is too weak to eliminate all bacteria, but at 37° C. in 60 per cent. strength all bacteria except spore-bearing species may be destroyed in a few days. Hence it is suitable for the purification when the virus is devoid of sporogenous bacteria. Addition of 1 per cent. phenol alone or 1 per cent. phenol and 60 per cent. glycerin both to the emulsion brings about a quicker elimination of bacteria, but this is accompanied by a more rapid deterioration of the virus itself. The same is true with the addition of one part per thousand of oil of cloves to 60 per cent. glycerin.

Recently Fornet (19), who has experimented with cultivation *in vitro* of vaccine virus, has recommended ether for removing the bacteria which do not possess spores. An emulsion of the virus can be freed of all bacteria except sporogenous ones by being shaken at room temperature for forty-eight hours with an excess of ether. The activity of the virus is, however, considerably diminished by ether treatment. By the ether method a vaccine pulp devoid of spore-bearing bacteria obtained as follows was secured free of all bacteria, and hence was suitable for cultivation in a pure state *in vivo*.

A sample[2] of glycerinated virus free from sporogenous bacteria was incubated at 37° C. for two days or longer, in order to destroy practically all the bacteria still present in it. The skin on the dorsal side of a rabbit was shaved and thoroughly cleansed with soap and rinsed with sterile distilled water. It was again shaved closely, after which the glycerinated virus was applied, in such strength as to produce separate eruptions. The vaccinated surface is protected from contamination by means of a sterile bandage. On the fourth or fifth day the bandage was removed and the surface washed first with absolute alcohol and then with ether. Several vesicles were selected and each was cleansed with 5 per cent. lysol solution and washed alternately with absolute alcohol and ether on sterile gauze. The eruptions were scraped with the edge of a sharp scalpel and the scrapings emulsified in several cubic centimeters of sterile saline solution. The emulsion was mixed with several volumes of ether and shaken in a sealed vessel for varying periods of time at room temperature. Samples were removed at the expiration of 1, 2, 4, 8, 12, 24, and 48 hours, from each of which cultures were prepared. When spores are absent sterility is usually obtained in twelve hours. The vaccinal activity falls at times to one-fifth of the original strength, but the characteristic properties remain unaltered.

TESTICULAR CULTIVATION IN RABBITS.

The vaccine emulsion so prepared is employed for the intratesticular inoculation of rabbits. Rabbits with well developed testicles should be chosen. After ether anesthesia an assistant holds the animal and fixes the testicles to prevent their withdrawal into the peritoneal cavity. The scrotal skin which is tightly stretched over the testicle is sterilized with 5 per cent. lysol solution and then painted with tincture of iodine. The operator next inserts the needle of a

[2] Several samples of the vaccine virus employed in the present investigation were furnished me by Dr. F. S. Fielder, Assistant Director of the Vaccine Laboratory of the Department of Health of the City of New York, to whom I wish to express my gratitude.

sterile syringe containing a 1 to 10 or 1 to 20 dilution of the emulsion into the testicular parenchyma along the long axis. The point of the needle is prevented from passing through the parenchyma to the tunica vaginalis. The contents are now gently forced out of the syringe into the organ at different regions by turning the direction of the needle. About one cubic centimeter of emulsion is injected into a testicle weighing from two to three grams. The organ is gently massaged to distribute the virus throughout the entire organ. The operation is practically painless.

The method just described is employed for the inoculation of the testicular strains of the virus, in which case an emulsion of testicle previously inoculated with the virus is used. The stock emulsion is prepared by grinding up the aseptically removed organs with sterile saline or 60 per cent. glycerin solution in the proportion of one gram of the tissue to two or three cubic centimeters of the fluid and any dilution of it; about 1 to 10 or 1 to 20 in saline is prepared for the purpose of the injection.

The testing for bacteria in the emulsion is an important point. Cultures are set up with plain and glucose bouillon, ascitic fluid with and without bouillon, and ascitic fluid with a piece of fresh sterile rabbit kidney with and without a layer of sterile paraffin oil. The cultures are incubated at 37° C. for three to four days, and then subcultures on plain and ascitic glucose slant agar, deep layer glucose agar, and ascitic tissue deep layer agar are made.[3] Film preparations stained by Gram or by Giemsa are also examined under the microscope, both from the cultures and from the testicles removed from the animal. In order to avoid occasional bacterial infection it has been found well to inoculate both testicles of each animal. The skin may be vaccinated at the same time. It is, moreover, advisable to use at least two rabbits for each transfer, because sometimes a rabbit reacts poorly to the vaccinal inoculation of both skin and testicle; but once the virulence of the virus has reached a certain height this precaution is no longer necessary.

[3] In the latter part of the present work initial culture in tissue bouillon and subcultures in glucose agar (deep) and plain agar (plate) were found to be sufficient.

RESULTS OF EXPERIMENTS WITH RABBITS.

As the accompanying table shows, the passage of the testicular virus from animal to animal offered some difficulty until it had been carried on for a number of generations. In this experiment success was not assured until the virus had been transferred about ten times. That this result was due to increase in virulence of the virus in the later generations may be deduced from the greater activity manifested when it was tested upon the skin, as well as from the greater severity of the reactions in the testicles themselves (table I).

TABLE I.

Date of transfer.	Generation.	Results.	Date of transfer.	Generation.	Results.
1914					
Jan. 29–Feb. 2	1	+	Sept. 28–Oct. 3	32	++
Feb. 2– 9	2	+	Oct. 3– 7	33	++
Feb. 9–14	3	+	Oct. 7–12	34	++
Feb. 14–22	4	+	Oct. 12–16	35	++
Feb. 23–27	5	+[4]	Oct. 16–20	36	++
Mar. 10–16	6	+[5]	Oct. 20–25	37	+++
Apr. 22–25	7	+[6]	Oct. 25–29	38	+++
May 1– 6	8	+	Oct. 29–Nov. 3	39	+++
May 25–29	9	+	Nov. 3– 7	40	+++
May 29–June 3	10	++	Nov. 7–12	41	+++
June 3– 6	11	++	Nov. 12–16	42	+++
June 8–12	12	++	Nov. 16–21	43	+++
June 12–15	13	++[7]	Nov. 21–26	44	+++
June 18–25	14	++	Nov. 26–30	45	+++
June 25–July 2	15	++	Nov. 30–Dec. 5	46	+++
July 2– 8	16	++	Dec. 5– 9	47	+++
July 8–13	17	++	Dec. 9–13	48	+++
July 13–16	18	++	Dec. 13–16	49	+++
July 17–20	19	++	Dec. 16–20	50	+++
July 20–23	20	++	Dec. 20–24	51	+++
July 26–30	21	++	Dec. 27–31	52	+++
July 30–Aug. 3	22	++	1915		
Aug. 4– 7	23	++	Jan. 4– 8	53	+++
Aug. 11–17	24	++	Jan. 8–12	54	+++
Aug. 17–22	25	++	Jan. 15–19	55	+++
Sept. 1– 4	26	++	Jan. 23–27	56	+++
Sept. 7–10	27	++	Jan. 31–Feb. 4	57	+++
Sept. 12–16	28	++	Feb. 8–12	58	+++
Sept. 16–20	29	++	Feb. 16–20	59	+++
Sept. 21–24	30	++	Feb. 24–28	60	+++
Sept. 24–28	31	++			

[4] From this material two further generations were carried on and then lost, so it was necessary to come back to this generation and to start again.

[5] From this two generations were carried on and lost, necessitating a return.

[6] Only one out of several rabbits gave good results.

[7] Two rabbits were inoculated with this material, but only one gave a satisfactory result.

The testicular strain indicated in the table has passed through sixty successive generations within twelve months. It is interesting to find that considerable resistance to testicular adaptation was exhibited at the seventh transfer, after which no serious obstacle was met in carrying on the passages.

TESTICULAR VACCINAL PROCESSES IN RABBITS.

In order to follow the course of the vaccinal processes in the testicular tissue the following experiments were carried out.

Thirteen male rabbits were inoculated into each testicle with 1 c.c. of a 1:20 dilution of the saline testicular emulsion derived from the rabbit inoculated with the thirtieth generation of the testicular strain and castrated under ether anesthesia on the fourth day.

The testicles of these animals were removed one after the other and tested successively for activity on the skin, cornea, and testicles of normal rabbits every twenty-four hours, over a period of eighteen days, and then after 3, 4, 5, and 8 weeks.

Table II shows the results obtained.

During the first twenty-four hours the testicle presents little change, except that microscopic foci of infiltration of polynuclear leucocytes and exudate are observed in the interstitial spaces (figure 32, compare with normal, figures 30 and 31). At the end of forty-eight hours the swelling and induration of the organ begin to increase rapidly and the testicle becomes congested and edematous (figure 2). The content of the virus, which was almost zero after twenty-four hours, now reaches about 100 times that found at the end of the first day. When examined in sections an enormous leucocytic infiltration is seen in the interstitial tissues, and some leucocytes are contained in tubules (figure 33). The testicular cells are hydropic and fill up the tubular lumen. At the end of three days the infiltration has increased in intensity and extent (figures 3 and 18, compare with normal, figure 17), and the vaccinal activity has risen to at least 300 times that present twenty-four hours and about three times that present forty-eight hours after the inoculation.

The external changes present in the four day specimen (figures 4 and 34) resemble those occurring in the three day specimen, except that the testicle is more compact and less elastic and the amount of

TABLE II.

Testicles removed after	Gross appearance of specimens.	Tests for activity of emulsions on rabbits.			
		Dilution of emulsion.	Skin.	Cornea.	Testicle.
24 hrs.	Almost no swelling; traumatic hemorrhages	1 : 10	1 eruption	−	+
		1 : 100	−	.....	.....
		1 : 1,000	−	.....	−
48 hrs.	Vascular injection, edema, and moderate induration (figure 2)	1 : 10	++	++	++
		1 : 100	+	−	.....
		1 : 1,000	<+	−	.....
		1 : 10,000	−	−	−
3 dys.	Marked induration, great congestion, greyish mottling, edema (figure 3)	1 : 10	++	++	++
		1 : 100	++	+	.....
		1 : 1,000	+	−	.....
		1 : 10,000	−	−	+
4 dys.	Severe congestion, edema, induration and swelling; numerous yellowish grey spots on the organ (figure 4)	1 : 10	Confluent	++	++
		1 : 100	+++	+	.....
		1 : 1,000	++	+	.....
		1 : 10,000	<+	−	++
5 dys.	Similar to the last (figure 1)	1 : 10	Confluent	++	++
		1 : 100	Confluent	++	.....
		1 : 1,000	++	+	.....
		1 : 10,000	+	?	++
5 dys.	Similar to the last (figure 5)	1 : 10	Confluent	++	++
		1 : 100	+++	+	.....
		1 : 1,000	++	−	.....
		1 : 10,000	−	?	++
5 dys.	Extensive hemorrhages; otherwise similar to the last	1 : 10	Confluent	++	++
		1 : 100	++	+	.....
		1 : 1,000	+	−	.....
		1 : 10,000	−	−	+
6 dys.	Marked induration, but somewhat less than on the previous day. Less edema (figure 6)	1 : 10	Confluent	++	++
		1 : 100	++	+	.....
		1 : 1,000	+	−	.....
		1 : 10,000	−	−	+
7 dys.	Induration somewhat less than on the preceding day. Yellowish grey specks prominent on the surface (figure 7)	1 : 10	++	++	++
		1 : 100	++	+	.....
		1 : 1,000	+	−	.....
		1 : 10,000	−	−	+
8 dys.	General induration disappearing; organ flabby; disseminated yellowish grey spots (figure 8)	1 : 10	+	++	++
		1 : 100	<+	−	.....
		1 : 1,000	−	−	.....
		1 : 10,000	−	−	−
9 dys.	Induration gone; soft and pale, slightly below original volume; some focal infiltration (figure 9)	1 : 10	+	+	++
		1 : 100	<+	−	.....
		1 : 1,000	<+	.....	.....
		1 : 10,000	−	.....	−
10 dys.	Induration gone; general atrophy; some focal induration (figure 10)	1 : 10	+	+	++
		1 : 100	<+	−	.....
		1 : 1,000	<+	.....	.....
		1 : 10,000	−	.....	−
11 dys.	Marked reduction in size; no induration (figure 11)	1 : 10	+	+	++
		1 : 100	<+	−	.....
		1 : 1,000	−	.....	.....
		1 : 10,000	−	.....	−
12 dys.	No induration, soft and pale, much smaller than original (figure 12)	1 : 10	+	+	+
		1 : 100	−	−	.....
		1 : 1,000	−	.....	.....
		1 : 10,000	−	.....	−

TABLE II.—*Concluded.*

Testicles removed after	Gross appearance of specimens.	Tests for activity of emulsions on rabbits.			
		Dilution of emulsion.	Skin.	Cornea.	Testicle.
13 dys.	Normal in size, pale and soft; some greyish spots	1 : 2	+	+	.....
		1 : 10	<+	.....	+
		1 : 20	−	−	.....
		1 : 100	−	.....	.....
14 dys.	Similar to the last	1 : 2	−	−	.....
		1 : 10	−	.....	+
		1 : 20	−	.....	.....
15 dys.	Small, pale, flabby; few greyish foci	1 : 2	+	+	.....
		1 : 10	<+	−	+
		1 : 20	<+	−	.....
16 dys.	Similar to the last	1 : 2	+	+	.....
		1 : 10	<+	−	+
		1 : 20	<+	−	.....
17 dys.	Marked atrophy	1 : 2	−	−	+
		1 : 10	−	−	.....
		1 : 20	−	−	.....
18 dys.	Similar to the last	1 : 2	−	−	−
		1 : 10	−	−	−
21 dys.	Small, fibrous; few minute foci of infiltration	1 : 2	<+	+	+
		1 : 10	−	−	.....
28 dys.	Similar to the last	1 : 2	1 eruption	−	+
		1 : 10	−	−	.....
35 dys.	Marked atrophy; small area of necrotic tissue present	1 : 2	−	−	−
		1 : 10	−	−	−
56 dys.	One testicle reduced to a small fibrous mass; the other less atrophied	1 : 2	−	−	−
		1 : 10	−	−	−

exudate in the tunica vaginalis and in the testicular tissue itself is more copious. The color of the testis has become purplish red, spotted here and there with irregular yellowish areas of different dimensions (figures 13 to 16). The organ is easily torn. The sections show numerous groups of several tubules each, which have lost the property of taking up the basic stain, indicating a total necrosis of the structures (figures 19 and 36). These changes are not universal, since areas exist in which no apparent serious alteration has taken place. At this stage the vaccinal power is nearly three times as great as it was at the end of three days, and at least 1,000 times as great as at the end of the twenty-four hour period. The changes in the five day specimen are about the same as those of the four day specimen. The vaccinal activity seems now to have attained its maximum height, since almost confluent eruptions on the skin of rabbits are yielded in dilutions of 1 to 1,000. The sections indicate beginning disintegration of the leucocytic elements and of

affected tubular cells (figures 5, 29, 29 a, and 35; compare with normal, figure 28).

At the expiration of six days the testicle has become of softer consistency (figure 6), and the edema and cellular infiltration have begun to recede. Microscopically foci of small round cells in the infiltrated areas and fibrin masses are distinguished (figures 37 and 38). The vaccinal activity is similar to that of the five day specimen. After seven days the resolution of the infiltration proceeds more rapidly (figures 7 and 20), while the vaccinal activity is less than that of the preceding day. From now on the testicles diminish rapidly in volume, so that the ten day specimen is below normal in size (figures 8, 9, 10, 21, and 22). The organs are now pale and of soft texture. The activity of the virus is much less than it was at the end of seven days. At the end of eleven days it is still weaker. From twelve to eighteen days the shrinkage of the organ continues and the sections show loss of testicular cells (figures 11, 12, 23 to 27, and 39 to 44) and collapse of the tubules. The virus has now disappeared wholly or almost wholly, and in some instances no vaccinal effect could be obtained in any concentration. Hence this period may be regarded as that of elimination of the virus, while the exact moment of disappearance probably depends upon the quantity of the virus originally developed and the degree of immunity displayed. At later periods, namely after five weeks, no virus could be detected, while the atrophy of the testicular parenchyma may be complete, so that a fibrous mass containing a few unusually thin tubules, lined with a single layer of epithelial cells may alone remain (figures 27 and 45).

The tunica vaginalis is often intensely infiltrated with polynuclear leucocytes (figure 46) and contains a considerable amount of the virus; apparently the virus multiplies here as it does in other epithelial cells. The epididymis shows a slight infiltration only during the early period.

Spermatogenesis ceases quickly and the various sperm cells quickly degenerate under the influence of the vaccinal process.

EFFECTS OF THE TESTICULAR VACCINAL STRAIN ON THE SKIN AND CORNEA.

The testicular strains of the virus were employed to inoculate the shaved skin and scarified corneal surfaces of rabbits. The effects of the vaccinations so carried out may be followed in table III. They will be recognized as characteristic of the effects produced by active vaccine virus as usually prepared in the calf. The microscopical features of the process are also typical, including the presence of the Guarnieri, or vaccine, bodies so called (figures 49 and 50).

TABLE III.

At the end of	Average course in rabbits.		
	Skin.	Cornea.	Temperature.[8]
24 hrs.	Diffuse reddening	Slight swelling	38.9°
48 hrs.	Mottled reddish areas	Slight turbidity	39.5°
3 dys.	Fairly defined flat erythema, in part confluent	Distinct turbidity	40.0°
4 dys.	In part confluent, mostly discrete raised papules with induration and areola	Advancing in degree and extent	40.8°
5 dys.	Distinct raised round vesicles with areola	Sometimes ulceration	40.2°
6 dys.	Development into pustular eruptions	Ulceration	39.8°
7 dys.	Pustules with scanty content; tendency to dry up	No change	38.8°
8 dys.	Inflammatory processes disappearing	Ulcer persists	38.9°
9 dys.	Stage of crust formation and desquamation	Ulcer persists	38.7°
10 dys.	Stage of crust formation and desquamation	Ulcer persists	38.8°

EFFECTS OF VARYING CONCENTRATION OF TESTICULAR VIRUS UPON THE SKIN, CORNEA, AND TESTICLE.

In order to determine the sensitiveness of the skin, cornea, and testicle to vaccine virus, emulsions of the testicular virus of different concentrations were prepared and inoculated into the several parts mentioned.

For this purpose a specimen of testicle representing the thirty-first passage or generation was employed. The dilutions in sterile saline were as follows: 1 to 10, 1 to 100, 1 to 300, 1 to 1,000, 1 to 3,000, 1 to 10,000, 1 to 30,000, 1 to 100,000. The skin and cornea were

[8] Average from fifteen rabbits.

inoculated in the usual manner, and 0.5 of a cubic centimeter was injected into the testicle.

The result is summarized in table IV and may be stated as follows: Up to a dilution of 1 to 300 the testicular emulsion causes marked vaccinal reactions in the skin, cornea, and testis. The skin surface still reacts slightly at the 1 to 1,000 dilution, but the cornea does not. Beyond the 1 to 1,000 dilution the skin reacts no longer, while the testicle continues to react even to dilutions of 1 to 100,000, the limit of the experiment. However, dilutions of 1 to 3,000 and higher retarded somewhat the testicular reaction, which in the end

TABLE IV.

Dilution of emulsion.	Reactions following the inoculation of different dilutions of the emulsion as indicated in the first column.			Tests of activity of emulsions prepared from testicles[9] which had been injected with different dilutions.			
	Skin.	Cornea.	Testicle.	Dilution of emulsion of each testicle.	Skin.	Cornea.	Testicle.
1 : 10	+++	+	3 dys. already marked induration and edema	1 : 100	+	+	++
				1 : 1,000	+	.....	++
1 : 100	++	+	3 dys. already marked induration and edema	1 : 100	++	++	++
				1 : 1,000	+	.....	++
1 : 300	++	+	3 dys. already marked induration and edema	1 : 100	++	++	++
				1 : 1,000	+	.....	++
1 : 1,000	+	−	3 dys. already marked induration and edema	1 : 100	++	++	++
				1 : 1,000	+	.....	++
1 : 3,000	−	−	3 dys. only slight induration; 5 dys. marked increase	1 : 100	++	++	++
				1 : 1,000	+	.....	++
1 : 10,000	−	−	3 dys. only slight induration; 5 dys. marked increase	1 : 100	++	++	++
				1 : 1,000	+	.....	++
1 : 30,000	−	−	3 dys. only slight induration; 5 dys. marked increase	1 : 100	++	++	++
				1 : 1,000	+	.....	++
1 : 100,000	−	−	3 dys. only slight induration; 5 dys. marked increase	1 : 100	++	++	++
				1 : 1,000	+	.....	++

was quite identical with that produced by the stronger concentration. In an experiment made somewhat later and after the testicular strain of the virus had been intensified by repeated passages, a skin reaction of small degree but of characteristic kind could be elicited in the 1 to 100,000 dilution.

This experiment serves also to bring out distinctly the fact of

[9] The testicles were removed from the animals on the sixth day after the inoculation.

the rapid multiplication of the virus in the testicle of rabbits, and is shown in the second half of table IV. The vaccinal effect upon the skin, cornea, and testicle became essentially the same in kind and degree, irrespective of the concentration of the virus emulsion injected originally into the testicle. At the expiration of six days the testicles inoculated with virus in the 1 to 100,000 dilution yielded a virus emulsion as active as the original testicular material employed for preparing the stock emulsion. The rapidity and ease of multiplication of the virus within the testicle is well illustrated by this experiment. The causes of the small variations in effect as indicated in the table as occurring between emulsions of testicles receiving the higher and lower dilutions of virus are not at once apparent. Possibly certain rabbits restrain the multiplication of the virus more than others; possibly concomitant immunity reactions come into play.

TESTICULAR CULTIVATION IN BULLS.

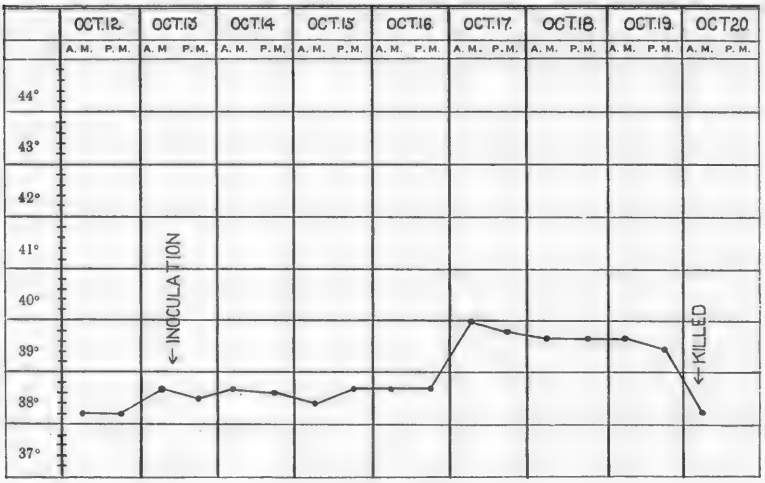
Experiments were next performed on the testicles of young bulls, with the view of ascertaining whether this species of animal would be suitable for use, both from the quantitative and qualitative relation of the virus, since it has long been customary to produce vaccine in the skin of this species.

Bull 1.—Weight, 750 pounds. The scrotal skin was shaved, washed with soap, and sterilized by sublimate alcohol. The vaccine virus employed for the injection consisted of testicular emulsion derived from the testicles of two rabbits which had been inoculated with the 30th generation of the testicular strain and castrated at the end of seven days. This emulsion produced a fairly thick eruption on the skin of rabbits in the dilution of 1:100. On Oct. 13, 1914, 10 c.c. of the emulsion diluted 1:100 were injected into the right, and 15 c.c. into the left testicle under aseptic precautions.

The testicles showed signs of swelling on the third day (forty-eight hours), and increased further in size and density up to the end of six days. The left testicle was much more swollen than the right. The temperature remained normal (38.7° to 38.9°) until the end of the fifth day, when it rose to 40.6°, 40.1° (6th day), and remained at 40.4° as late as the end of the sixth day. On the morning of the eighth day the temperature was 38.7° (text-figure 1).

The animal was slaughtered and the testicles were removed under aseptic precautions on the eighth day. The subcutaneous tissue and the tunica vaginalis were edematous and hemorrhagic, especially along the needle track. The testicles themselves were also edematous and showed a mass of coagulated blood at the site where a blood vessel must have been injured by the needle puncture.

Compared with the normal the vaccinated testicles were firmer and more pinkish in color, besides showing numerous ecchymoses. The left testicle was more altered than the right; the former weighed 310 and the latter 250 gm.



TEXT-FIG. 1. Bull 1.

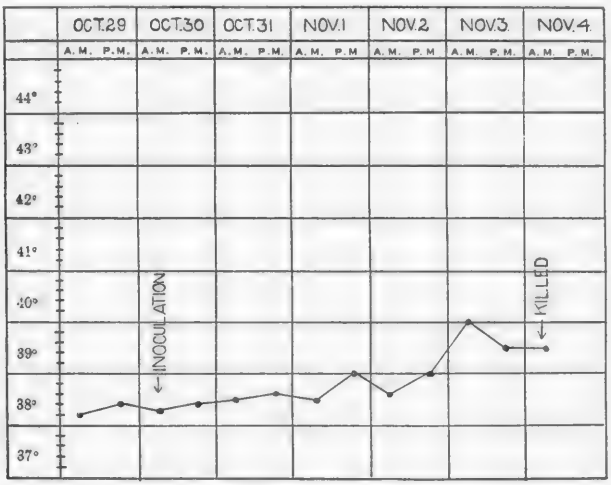
Emulsions in sterile saline solution were made with the tissues immediately about the point of insertion of the needle and also of portions remote from the site of injection. The tests for activity were made upon the shaved skin, cornea, and testicles of rabbits.

TABLE V.

Bull 1.	Dilution of emulsion.	Tests on rabbits. Skin.	Cornea.	Testicle.
Emulsion of tissue around site of injection.....	1 : 10	+++	++	++
	1 : 100	+	+	++
	1 : 1,000	<+	−	++
	1 : 10,000	−	.....	++
Emulsion of tissue remote from site of injection..	1 : 10	++	++	++
	1 : 100	+	−	++
	1 : 1,000	−	.....	−
	1 : 10,000	−	.....	−
Exudate in tunica vaginalis.....	Undiluted	−	−	−
	1 : 10	−	−	−

The exudate contained in the tunica vaginalis was likewise tested. Both exudate and emulsions were found sterile for bacteria. Table V summarizes the results, which show that the virus multiplied to a much smaller extent in the testicular tissues of the bull than is the rule in the testicle of the rabbit. That the virus multiplied in some degree is highly probable; and that the edematous fluid in the tunica vaginalis was devoid of virus is an interesting point.

Bull 2.—Weight, 680 pounds. Oct. 30, 1914. The left testicle was injected at four different sites in order to distribute the virus widely with a 1:100 dilution of rabbit testicular virus in the thirty-third generation, the total quantity inoculated amounting to 20 c.c. At the same time the skin of the ventral surface below the navel was shaved, scarified, and inoculated with some of the same emulsion. The inoculated testicle after forty-eight hours was tense on palpation, distinctly swollen, and indurated. The swelling and induration increased during the next three days. The skin showed distinct papules three days after the inoculation, which became typical pustules two days later (figure 53). The temperature rose to 40° to 40.7° on the fourth day, and was 40° on the sixth day. The bull was slaughtered on the sixth day and the testicles were aseptically removed (text-figure 2).



TEXT-FIG. 2. Bull 2.

The scrotum was found to be edematous and infiltrated, but no adhesion was present between the tunica and the testicular surface. The moderate amount of serous and hemorrhagic exudate proved to be sterile for bacteria. Upon section

serous exudate oozed freely from the testicle. The points of injection could be detected by the presence of numerous minute hemorrhages within areas which were distinctly firmer than the surrounding parts. Coagula of blood occurred here and there (figures 54 and 55). The uninoculated right testicle showed no change. The weight of the left testicle was 229 and that of the right 199 gm.

Emulsions were prepared both from the tissues immediately about the sites of injection and from parts remote from them. The tests were made on the skin, cornea, and testicles of rabbits.

TABLE VI.

Bull 2.	Dilution of emulsion.	Tests on rabbits. Skin.	Cornea.	Testicle.
Emulsion of tissue around zone of injection.	1 : 10	Confluent	++	++
	1 : 100	++	+	++
	1 : 1,000	+	.....	++
	1 : 10,000	<+	.....	++
Emulsion of tissue remote from site of injection	1 : 10	++	++	++
	1 : 100	+	+	++
	1 : 1,000	<+	.....	+
	1 : 10,000	−	.....	−
Exudate in tunica vaginalis	Undiluted	<+	+	+
	1 : 30	−	−	−

Table VI, which summarizes the results, indicates that the tissues about the sites of inoculation contain an amount of the virus about equal to that of rabbit's testicles inoculated with similar material, and that the degree of activity possessed by the two testicular strains is about the same. On the other hand, the emulsion made from the remote portions of the testicle produced far less effect, although it was still active up to the dilution of at least 1 to 1,000. The exudate in the tunica contained a trace of the virus, but this may have been due to a contamination of this exudate in the opening of the testicle.

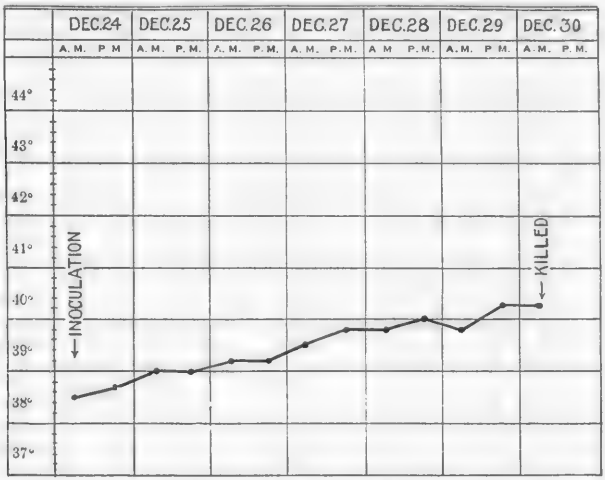
Bull 3.—Weight, 720 pounds. Nov. 11, 1914. Both testicles were inoculated with an emulsion of a testicular strain obtained from a rabbit inoculated with the testicular strain of bull 2. The emulsion was, however, contaminated with a *xerosis* bacillus. The local and general reactions were similar to those described in the previous two bulls. In spite of the admixture of *xerosis* bacilli with the virus the local reaction was not different from those following the injection of a pure virus. The animal was slaughtered on the ninth day after injection.

The testicular tissues were edematous, showed several well localized areas of

a greyish white color, and some hemorrhages caused by the trauma of the needle. The tissues were not softened. Cultures made from the emulsions of the testicles showed the presence of a few *xerosis* bacilli, but no other bacteria.

The vaccinal activity as tested on the rabbit showed that it was much weaker than that of bull 2.

Bull 4.—Weight, 140 pounds. Dec. 24, 1914. 4 c.c. of a 1:40 dilution of an emulsion of the fiftieth generation of testicular rabbit virus were injected into the left, and 3 c.c. of a 1:10 dilution of the testicular virus from bull 2 were injected into the right testicle. The organs were examined every twenty-four hours, but no particular difference was noticed on the two sides. Both showed marked induration within thirty hours. On the fifth day the swelling appeared to have reached the maximal stage. The temperature rose after three days and reached 40.8° on the sixth day. The animal was slaughtered at the end of six days (text-figure 3).



TEXT-FIG. 3. Bull 4.

The testicles had increased in volume, especially the one on the left side injected with the bull-rabbit strain; they were markedly edematous and congested. On section numerous greyish yellow specks were seen to be scattered throughout the organ, and were especially numerous along the sites of injection. Similar greyish yellow areas, it will be recalled, occur almost constantly in the inoculated rabbit's testicles.

The emulsions were made from both testicles separately and tested for their vaccinal activity, together with the controls of the standard glycerinated vaccine issued by the Department of Health of New

York City. The testicular emulsions were free of bacteria; the Health Department vaccine, of course, was not.

TABLE VII.

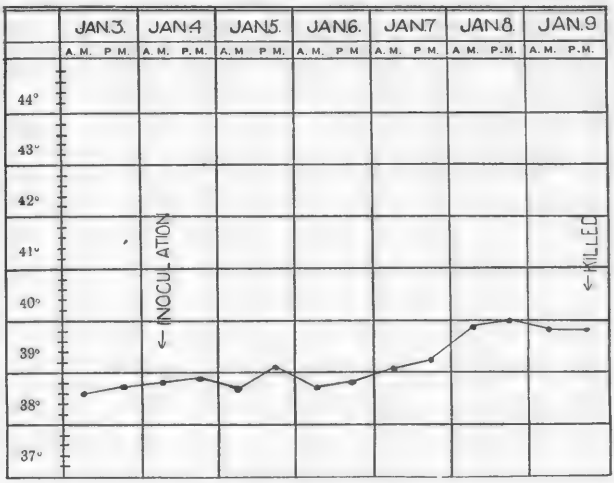
	Bull 4.	Dilution of emulsion.	Tests on rabbits.			Tests on calves.
			Skin.	Cornea.	Testicle.	Skin.
	Emulsion of testicle inoculated with bull-rabbit testicular strain	1 : 10	Confluent	++	++	Confluent.
		1 : 100	Confluent	+	++	++
		1 : 1,000	+	±	++	+
		1 : 10,000	<+	−	++	<+
	Emulsion of testicle inoculated with bull-rabbit testicular strain no. 2	1 : 10	Confluent	++	++	Confluent.
		1 : 100	Confluent	++	++	Almost confluent.
		1 : 1,000	++	+	++	++
		1 : 10,000	+	−	++	<+
Regular vaccine as controls	N. Y. D. H. standard vaccine 1st sample	1 : 10	Confluent	++	++	Confluent.
		1 : 100	Confluent	++	++	Almost confluent.
		1 : 1,000	++	+	+	++
		1 : 10,000	<+	−	<<+	<+
	N. Y. D. H. standard vaccine 2d sample	1 : 10	Confluent	++	++	Confluent.
		1 : 100	++	+	+	++
		1 : 1,000	+	−	−	+
		1 : 10,000	−	−	−	−

The above experiments (table VII) demonstrate that a strong bacteria-free vaccine virus can be produced in the testicular tissues of the young bull by injecting the testicular strain derived from a bull or from a rabbit, and they also show that the vaccinal activity reaches the same strength as that possessed by a standard virus propagated on the skin of a calf. Moreover, no differences were noted in the type and course of the vaccinal processes as produced in the skin of rabbits and calves by the vaccines from the several sources. The only distinctions are quantitative ones, since the skin virus is more quickly diluted beyond effective strength than the testicular virus.

The next experiment was devised to compare the effect of the testicular inoculation of a testicular and a skin strain of vaccine, respectively, into the same calf.

Bull 5.—Weight, 160 pounds. Jan. 4, 1915. 3 c.c. of a 1 : 10 dilution of an emulsion of the testicular strain derived from bull 4 were injected into the right, and 3 c.c. of a 1 : 10 dilution of the New York City Department of Health standard vaccine into the left testicle. The next day the swelling and injection of the right testicle were marked, while the left was far less altered. This difference

became more pronounced, and on the sixth day only the right side presented the typical reaction, swelling, and induration previously noted, while the left was but slightly enlarged. The animal was slaughtered at the end of five and one-half days (text-figure 4).



TEXT-FIG. 4. Bull 5.

Upon exposing the testicles it was found that the left showed infiltration in small amount and only about the site of the injection, while the right was edematous throughout, greatly enlarged, and dotted with numerous minute foci of greyish yellow appearance. While the consistency of the left testicle which weighed 6.5 gm. was about normal, that of the right, which weighed 8 gm., was diminished.

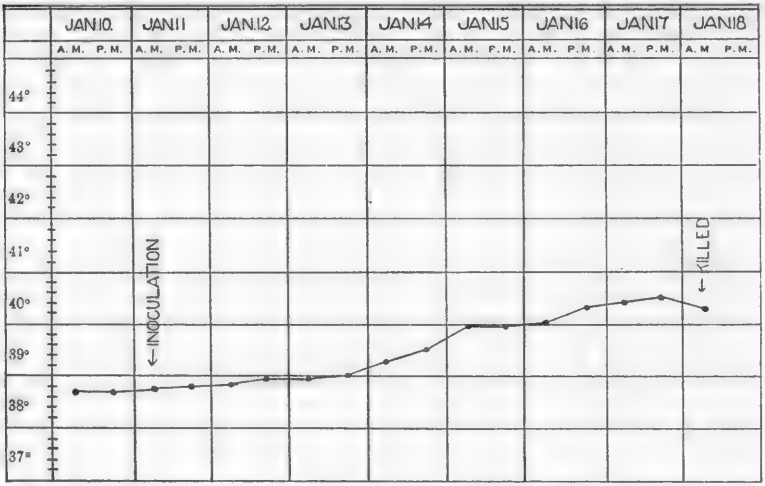
The vaccinal strength of both testicles was tested on the rabbit.

TABLE VIII.

Bull 5.	Tests on rabbits.			
	Dilution of emulsion.	Skin.	Cornea.	Testicle.
Emulsion of testicle inoculated with calf testicular strain no. 4	1 : 10	Confluent	++	++
	1 : 100	++	++	++
	1 : 1,000	+	−	++
	1 : 10,000	−	−	++
Emulsion of testicle inoculated with skin strain (N. Y. D. H.)	1 : 10	+	+	+
	1 : 100	<+	−	−
	1 : 1,000	−	.....	−
	1 : 10,000	−	.....	−

As table VIII indicates, the local reaction and the vaccinal activity were more marked in the case of the testicular than of the skin strain, from which it may be inferred that adaptation of the strain to the testicle is necessary as a prerequisite to the production by this means of strong vaccine.

Bull 6.—Weight, 225 pounds. Jan. 11, 1915. The left testicle was injected with an emulsion of the testicle that had been inoculated with the standard skin strain of vaccine, as mentioned above, and the right testicle with an emulsion of the testicle previously inoculated with a testicular strain. The local and general reactions were similar to those observed and described, except that the left testicle, weighing 9 gm., showed far less change than the right, which weighed 12.5 gm.



TEXT-FIG. 5. Bull 6.

(figures 56 and 57). The bull was slaughtered at the end of five and one-half days, and the vaccinal activity of each testicle was tested on the rabbit (table IX and text-figure 5).

Histological Changes.—The histological changes observed in the testicles of bulls inoculated with testicular strains of vaccine virus are similar to those enumerated in these organs of the rabbit. An acute inflammatory reaction arises, in which many polymorphonuclear leucocytes invade the interstitial tissues together with a serous exudate; the adjacent tubules are compressed by pressure of the exu-

TABLE IX.

Bull 6.	Tests on rabbits.		
	Dilution of emulsion.	Skin.	Cornea.
Emulsion of left testicle inoculated with skin strain.	1 : 10	+	+
	1 : 100	<+	−
	1 : 1,000	−	.....
	1 : 10,000	−	.....
Emulsion of right testicle inoculated with testicular strain	1 : 10	+++	++
	1 : 100	++	+
	1 : 1,000	+	.....
	1 : 10,000	−	.....

date, which also invades them, and the epithelial cells degenerate. The reaction is multifocal and affects parts remote from the site of inoculation, although the severer effects occur adjacent to the inoculation. Hemorrhages occur also (figures 57 to 66).

IDENTITY OF THE SKIN AND TESTICULAR STRAINS OF VACCINE VIRUS.

It may now be considered as established that vaccine virus derived from the specific skin lesions is capable of multiplying indefinitely in a pure state within the testis of the rabbit and the bull. There would appear to be no necessity for restoring the virus to skin surfaces in order to continue the propagation in the testicles. The testicular epithelium, once the virus is adapted to it, seems to provide as suitable a medium for its multiplication as the skin epithelium. Moreover, in order that the virus should propagate in the testis no previous injury of the organ is necessary, save that caused by the injection, so that in this respect the testicle may be regarded as exceeding the skin surface in susceptibility to its presence. Possibly anatomical structure accounts for this difference; unless scarified the virus has no opportunity of adhering to and entering intimately into relation with the skin surface, while injection into the testicle brings the virus at once into immediate proximity to the tissues composing that organ.

The question which arises is whether the skin and testicular strain of virus remain identical in physiological properties. One has, of course, been derived from the other; but it remains to be considered whether in the course of adaptation the testicular strain has undergone modification.

Several criteria can be employed to decide this point: (a) type and course of the eruption of the skin of susceptible hosts (rabbit, calf, man); (b) characteristic lesion of the cornea of the rabbit; (c) histological processes within the lesions caused by the virus; (d) phenomena of immunity arising after vaccination; (e) resistance to the action of certain agencies, such as glycerin, ether, chloroform, phenol, drying, and heating.

It may be assumed that merely completely freeing vaccine virus from the usual bacteria which contaminate it in practice will not change its physiological properties. This probability is indicated by the effect of glycerination which reduces the bacterial content. The practical advantage of this reduction is obtained in milder vaccinal effects in man, which are equally protective with the severer effects resulting from bacterial coöperation.

Hence it may be hoped that the complete removal of the contaminating bacteria should still further improve the results of vaccination in that the secondary operation of bacteria contained within the virus will be entirely eliminated. The employment of such a pure strain virus will, at the same time, do away entirely with the potential danger of contamination of the virus with tetanus bacilli or spores, a subject which has always engaged the attention of producers, and respecting which special precautions are taken.

Skin Reactions.—Both skin and testicular strains of virus were inoculated simultaneously on different parts of the shaved skin surfaces of rabbits and calves, after which the course of the vaccination was followed. No differences were noted (figures 67, 68, and 69).

Cornea.—The rabbit's cornea inoculated with skin and testicular virus, respectively, undergoes the same series of changes, and in each case Guarnieri bodies are produced. The results are indistinguishable (figures 49 and 51).

Histological Lesions.—When the testicular strains of virus are reimplanted upon the skin of the rabbit and calf, and the minute histological changes produced are compared with those caused by the skin strains of virus, no essential differences have been detected (figures 47, 48, 50, and 52). Moreover, the microscopical changes in the testicles produced by skin or adapted testicular strains of virus are essentially identical.

IMMUNITY PHENOMENA.

Considered from the practical standpoint the most important effect of vaccine virus is the immunity to reinoculation that it confers. Hence, experiments were conducted with the testicular strains in order to determine the immunity effects which it produces.

Experiment I.—Rabbit. First vaccination, Oct. 5, 1914, at three separate places on the shaved skin, with regular, glycerinated stock virus. Confluent vaccinal eruptions were produced.

Second vaccination, Feb. 12, 1915. The three places previously vaccinated were now revaccinated as follows: (a) an area with some of the virus used previously; (b) an area with rabbit testicular strain; (c) an area with bull testicular strain. The three areas reacted in identical manner: each area became congested, but neither vesicles nor papules appeared. The reddening of the skin was probably allergic in origin (20).

The control rabbit developed confluent eruptions from each of the three samples of virus.

Experiment II.—Rabbit. First vaccination, Oct. 5, 1914, at three separate places on the shaved skin, with a testicular strain of vaccine virus from a rabbit. Confluent vaccinal eruptions were produced.

Second vaccination, Feb. 12, 1915. The three places previously vaccinated were revaccinated as follows: (a) an area with some of the virus used previously; (b) an area with bull testicular strain; (c) an area with New York City Department of Health vaccine. On the following day slight transient scattered erythema was noted, but neither papules nor pustules.

The control rabbit developed typical confluent eruptions from each of the three samples of virus.

Experiment III.—Rabbit. First vaccination, Feb. 2, 1915, at three places on the shaved skin, with testicular strain from a bull calf (No. 4). Confluent vaccinal eruptions were produced.

Second vaccination, Feb. 17, 1915. The three places previously vaccinated were revaccinated as follows: (a) an area with some of the virus used in the first vaccination; (b) an area with a rabbit testicular strain; (c) an area with the regular New York City Department of Health vaccine. Only moderate allergic reactions were observed.

Two other rabbits similarly tested gave identical results with the above experiment, except for the presence in one of them of a slight erythema along the scarified lines, which lasted three days.

Controls on a normal rabbit showed that the strength of the vaccine strains employed for the foregoing experiments was such as to produce confluent eruptions.

The experiments described are conclusive in demonstrating that the skin and testicular strains of vaccine virus yield identical immunity reactions both as regards kind and degree.

TESTICULAR CULTIVATION AS A MEANS OF PROPAGATION OF PURE VACCINE VIRUS.

It is obvious that a method of propagating the vaccine virus free from bacterial contamination would remove all danger of accidental infection through the introduction at the time of vaccination of an impure preparation containing certain bacteria. Without stopping to consider how often severe effects have resulted from bacterial contamination of the virus it may be urged that the use of a pure preparation will altogether preclude the possibility of a mixed infection from this source. The indefinite propagation of a highly potent vaccine virus in the testicular tissues of animals is comparatively a simple procedure, so that by means of a strictly aseptic technique combined with careful bacteriological control of the product, it will now become a simple matter to supply the medical profession with an absolutely pure virus, for the purpose of vaccination. As is well known, the method hitherto practised for propagating vaccine consists in utilizing the emulsion of the skin pulp from vaccinated calves. The usual fresh pulp contains many and various bacteria, so that to free the pulp of them it must be left in contact for a period with 60 per cent. glycerin or 1 per cent. phenol, before it is regarded as ready for use on human subjects. Since the strength of the virus also deteriorates with time the process of vaccine preparation has not been perfected from the practical point of view. On the other hand, a pure virus should be ready for use as soon as the usual bacteriological and potency tests are completed, that is, within about one week after the preparation of the emulsion of the testicle containing it.

The introduction of a pure vaccine into the public health service would be welcome, even if it were somewhat more expensive to produce than the usual vaccine. As a matter of fact it appears to be even more economical than the latter, as the following calculation shows.

A male rabbit weighing about 3 kg. yields from 7 to 10 gm. of testicular material containing the pure culture of vaccine. When this amount of tissue is emulsified in from 15 to 20 c.c. of 50 per cent. sterile glycerin, it will yield from 20 to 28 c.c. of a finished product of high potency. A sample of this product is diluted 1 : 1,000 and tested on the skin of rabbits. If a confluent eruption results,

which may reasonably be expected, the stock suspension may be further diluted with four times its volume of sterile 50 per cent. glycerin, since a sample of glycerinated virus which produces a confluent eruption in a dilution of 1:200 is sufficiently strong for use in man. Hence a single rabbit may yield 125 c.c. of finished glycerinated virus, or even more.

The average yield from the calf, according to Fielder,[10] is put at 40 to 100 gm. of fresh pulp, which emulsified with four parts of 50 per cent. glycerin gives 250 to 300 c.c. of vaccine virus ready for use within three months.

A comparison of the cost is in favor of the rabbit vaccine. For the preparation of 1,000 cubic centimeters of the finished vaccine product about 40 rabbits or 4 calves would be required. The initial cost of the former would be considerably smaller.

Moreover, the employment of rabbits instead of calves has advantages of another kind from those which have just been emphasized. The prevalence of foot-and-mouth disease among cattle has closed the market in many states to calves, so that the propagation of vaccine virus in the ordinary way has recently suffered. No such interruption is to be feared with respect to the use of rabbits. During the prevalence of such an epidemic it becomes essential also to keep the calves intended for vaccination under observation before use for a sufficient period to insure their freedom from foot-and-mouth disease infection.

Finally, it may be added that the bull may also come to be used for the testicular cultivation of the virus. However, the method has not yet been worked out for this animal as completely as it has for the rabbit. Hence it must be left to the future to determine whether the bull is strictly suitable for the purpose. This part of my work suffered interruption because of the prevailing epidemic of foot-and-mouth disease and the resulting quarantine upon cattle.

STANDARDIZATION OF VACCINE VIRUS.

Several methods are employed for determining the efficacy and potency of vaccine virus before distributing it for general use. The accepted methods agree in using animals before permitting the virus to be used on man. Theobald Smith recommends the skin of the calf, and requires that the eruptions produced there must correspond with those of the skin of a child. Calmette and Guérin (9, 21) and

[10] On the other hand, Rosenau (1) gives the amount of fresh pulp from a calf as 20 to 40 gm., which will yield 50 to 120 c.c. of the glycerinated emulsion.

Camus (22) employ the skin of the rabbit; while Chaumier (23) and others[11] first employ animals, and then themselves observe the vaccination effects on a few children before issuing the virus. There is agreement among the authorities that the skin of the rabbit and calf are reliable indications of the effects produced on human subjects.

Henseval and Convent (24) consider that a preparation which produces on the skin of the rabbit almost confluent eruptions in a dilution of 1 to 500 should be considered a suitable vaccine. Such a sample always causes an uninterrupted eruption along a line (linear eruption) on the skin of a child. They recommend as still usable even a weaker specimen provided it produces a continuous eruption in a 1 to 100 dilution.

The testicular vaccine strains which the author prepared in rabbits either fulfill or exceed the above requirement, since a few samples produced confluent eruptions in dilutions of 1 to 10,000. On the other hand, the products from the testicles of the bull were less active, and a few only of the preparations reached the lower standard.

PURE VACCINATION IN MAN.

The tests which were carried out in detail and with minute care in animals with the pure testicular strains of vaccine virus all indicated that the virus would produce typical vaccinal effects in the human subject.

Several adults, chiefly physicians familiar with the cultivation experiments, immediately volunteered for vaccination, which was carried out by the linear and scarification methods. Among the tests, which were all revaccinations, some were positive and others negative. In the positive results the vaccinal eruption was typical and no secondary infection arose to complicate or intensify the vaccination.

Among those in whom the vaccination was positive was a physician. After his experience he vaccinated his own child, three years and two months old, who had not previously been successfully

[11] The Department of Health of the City of New York issues only the preparations which have produced on primary vaccination cases fifteen typical takes on fifteen insertions made on five children, thus requiring 100 per cent. of positive takes for each sample of vaccine.

vaccinated. The linear eruption appeared on the fifth day, increased in intensity during the three succeeding days when it reached the maximum, and then receded. No pain or itching was complained of, and the highest temperature was 37.5° C., which was noted on the eighth day. On the fourteenth day scab formation was well advanced. I am indebted to the father of the child for the two photographs (figures 70 and 71) which illustrate the vaccinal effects on the fifth and sixth days.

SUMMARY.

Vaccine virus freed from all associated bacteria by means of suitable disinfecting agents can be propagated in a pure state in the testicles of rabbits and bulls. The virus cultivated in this manner is not only devoid of all bacteria, but appears capable of indefinite transfer from one animal to another. Sixty passages in rabbits of a pure strain have been made within one year.

Several transfers from testicle to testicle are required to bring about accurate adaptation of the virus to the testicular parenchyma, so that continued propagation in this way can be certainly secured. During the first transfers from testicle to testicle the activity of the virus may be less than the original skin specimen from which the pure strain was derived; but as the transfers proceed the activity rises until, when the adaptation is complete, the activity of the testicular equals that of the skin strain.

The multiplication of the virus within the testicle is maximum on the fourth or fifth day after inoculation; the quantity of virus remains about stationary until the eighth day, when diminution begins. At the expiration of five weeks no more virus could be detected in the testicle.

The vaccinal processes in the skin, cornea, and testicle of rabbits are practically identical whether the virus employed for the inoculation has been the original skin strain or the pure testicular strain; and the skin lesions produced in the calf with the two strains are also identical.

In conformity with the finding mentioned in the last paragraph it has been found that human beings react to the pure testicular strain of vaccine virus in an entirely typical manner. In the case

both of original vaccination and revaccination the vaccinal effects cannot be distinguished from those arising from uncomplicated skin virus.

Pure strains of testicular virus are readily produced, and once secured they may be propagated in a pure state by the method described in rabbits or bulls without difficulty and with economy. The pure strains thus obtained should supply an ideal form of virus for employment in the vaccination of human beings.

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EXPLANATION OF PLATES.

Plate 39.

Fig. 1. The right testicle shows swelling due to the injection of pure vaccine virus made five days previously; the left side is normal.

Figs. 2 to 12. The conditions of the testicles at various stages in the vaccinal processes produced by means of a pure vaccine virus, as follows: 2, 48 hours; 3, 3 days; 4, 4 days; 5, 5 days; 6, 6 days; 7, 7 days; 8, 8 days; 9, 9 days; 10, 10 days; 11, 11 days; and 12, 12 days after the inoculation. The yellowish grey mottles of irregular dimension can be seen in the specimens shown in figures 4, 5, and 6. The marked atrophy of the organ is easily noticeable in the specimens shown in figures 9, 11, and 12. All natural size.

Plate 40.

Fig. 13. A testicle four days after inoculation with pure vaccine virus. It shows marked vascular injection, edema, and several small yellowish grey spots visible on the surface.

Fig. 14. The organ on section, in which several lobules can be seen undergoing necrosis, as indicated by the greyish yellow, somewhat indurated areas. The parenchyma has bulged out of the tunica albuginea on account of the edema.

Figs. 15 and 16. Two specimens of testicles at the height of the vaccinal processes, one (figure 16) with hemorrhagic reactions. In the latter instance the necrotic changes are much more pronounced, as is indicated by the general yellowish grey color of the lobules on section.

Plates 41 and 42.

Fig. 17. The structure of the testicle of a normal rabbit under a low power (magnified 13 diameters).

FIG. 18. Diffuse interstitial infiltration of the vaccinated testis after three days. The compression of the tubules through intertubular edema and infiltration is well shown.

FIG. 19. A four day specimen which presents a large, irregularly defined infiltration and the destruction of the tubules.

FIGS. 20 to 24. Gradual absorption of the inflammatory products with distinct diminution of tubules. There are still many foci of infiltration.

FIGS. 25 and 26. The structure of testicles which had been vaccinated three and seven weeks previously.

FIG. 27. A longitudinal section of a testicle which shrank as the result of vaccination fifty-six days previously. There are only a few tubules left, all without any spermatogenetic activities. Magnification: ×13 throughout.

PLATE 43.

FIG. 28. A section of normal rabbit's testicle stained by the Borrel-Calkin method (25).

FIGS. 29 and 29 a. The peculiar round or oval bodies of varying size within the affected cellular elements in the vaccinated testicles of rabbits. These bodies appear brilliantly red when stained by the Borrel-Calkin method, and they resemble the vaccine bodies so called. These bodies may have been derived from the nuclei of the cells under the influence of the vaccine virus. Magnification: × 340 throughout.

PLATE 44.

FIGS. 30 and 31. The structure of a normal testicle of the rabbit.

FIG. 32. An early polynuclear infiltration of the interstitial spaces of a vaccinated testicle of the rabbit after twenty-four hours.

FIG. 33. A similar but more advanced stage after three days.

FIG. 34. Extensive involvement of the testicular tubules as well as the interstitial tissues after four days.

FIG. 35. A focus where polynuclears are seen to have begun to break up after five days.

FIG. 36. An area of extensive necrosis occurring in a specimen removed after four days.

FIG. 37. The cellular infiltration and fibrin in a specimen after five days (Weigert stain).

FIG. 38. An intratubular infiltration occurring in a specimen seven days after inoculation. The cells are basophilic leucocytes. Magnification: × 170 throughout.

PLATE 45.

FIG. 39. A tubule completely filled with leucocytes in a specimen eight days after inoculation.

FIGS. 40 and 41. Enormous cellular infiltration along the tunica albuginea in nine day specimens.

FIG. 42. The detachment of the degenerated testicular cells from the wall of affected tubules in a twelve day specimen.

FIG. 43. A focus of infiltration in a twenty-one day specimen. Such a focus is rather rare in old specimens.

FIG. 44. A focus of infiltration which was found in a fifty-six day specimen.

FIG. 45. The general changes of the structure of the vaccinated testicle after fifty-six days.

FIG. 46. The infiltration of the tunica vaginalis in a rabbit with polynuclear leucocytes in a four day specimen.

FIGS. 47 and 48. The vaccinal reactions on the skin of rabbits. Magnification: × 170 throughout.

PLATE 46.

FIG. 49. The Guarnieri, or vaccine bodies, so called, in the corneal epithelium of a rabbit inoculated with pure testicular vaccine virus. Three day specimen.

FIG. 50. The vaccinal reaction in the skin of a rabbit inoculated with the testicular strain of vaccine virus. Stained with Calkin's modification of Borrel's method.

FIG. 51. The vaccine bodies in a rabbit's cornea inoculated with regular vaccine strain.

FIG. 52. Normal rabbit skin stained with the Borrel-Calkin method.

PLATE 47.

FIG. 53. The inoculated (left) and normal (right) testicles of bull 2 on the sixth day after the injection of pure testicular vaccine from a rabbit. The left side is seen to be somewhat larger than the right. The skin shows typical vaccinal pustules also produced with the same material.

FIG. 54. The inoculated testicle and the tunica vaginalis of the same animal, the latter showing much hemorrhage.

FIG. 55. The same organ on section. One notices the hemorrhagic foci and necrotic area.

FIGS. 56 and 57. The testicles of bull 6. The larger one was inoculated with the testicular, and the smaller with a skin strain of vaccine virus, and both were removed on the sixth day.

PLATE 48.

FIG. 58. The structure of a normal testicle of a young bull. One recognizes at once that the spermatogenesis is in full display.

FIGS. 59 to 61. The reactions following the injection of pure testicular vaccine strain into the organ. They represent changes found on the sixth day.

FIGS. 62 to 66. The vaccinal processes in the testicles of bull calves. The changes are similar to those found with young bulls and rabbits, except that the animals were too young to have any spermatogenesis, even in the unaffected areas of the organ. Both in the young bulls and in the bull calves the interstitial infiltration with polynuclear leucocytes is marked. Magnification: × 170 throughout.

PLATE 49.

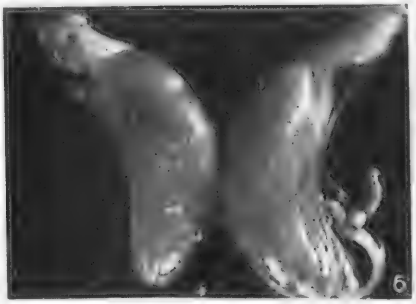
FIG. 67. The typical vaccinal effect on the skin of a rabbit inoculated with a pure testicular strain of vaccine virus on the sixth day.

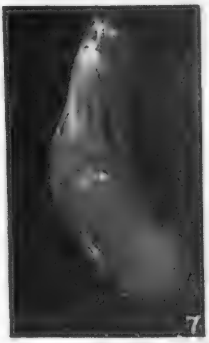
FIG. 68. A confluent eruption on the skin of a rabbit vaccinated with a pure testicular strain.

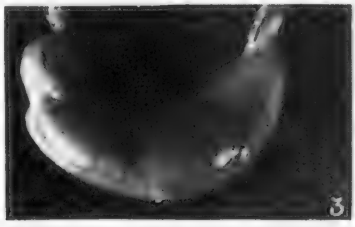
FIG. 69. The typical vaccinal effect on the skin of a calf inoculated with a pure testicular strain of vaccine virus obtained from a rabbit, on the sixth day. Natural size.

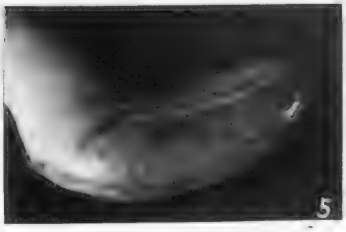
PLATE 50.

FIGS. 70 and 71. The fifth and the sixth day phases of the first vaccination in a child three years and two months old. The virus was prepared in the rabbit according to the method described in this work, and was absolutely free from bacteria. Notice the uninterrupted linear eruption on both sides. In figure 71 the mottled appearance around the site of vaccination is due to the adhesion of the vaccine shield.









(Noguchi: Cultivation of Vaccine Virus.)

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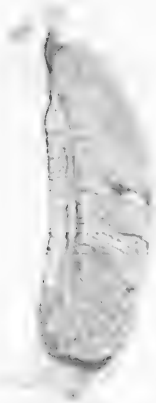

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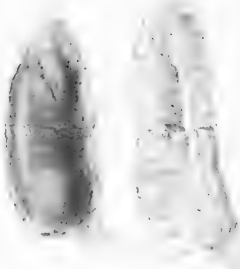

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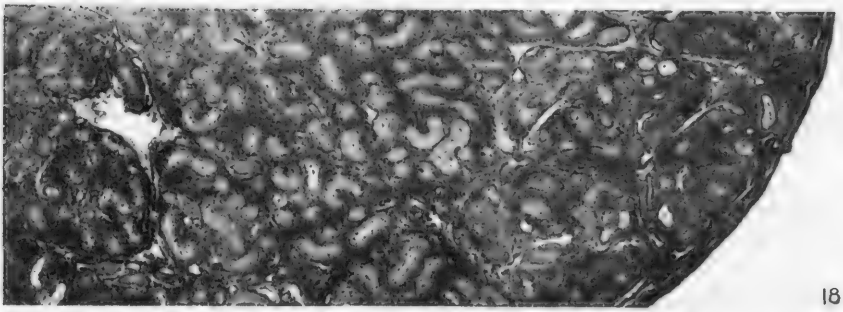

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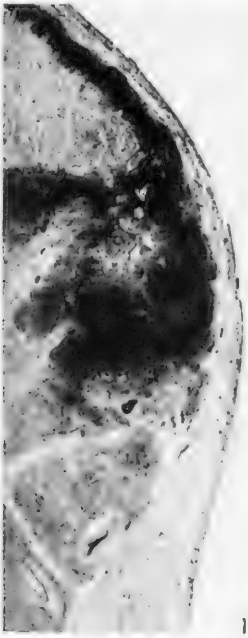

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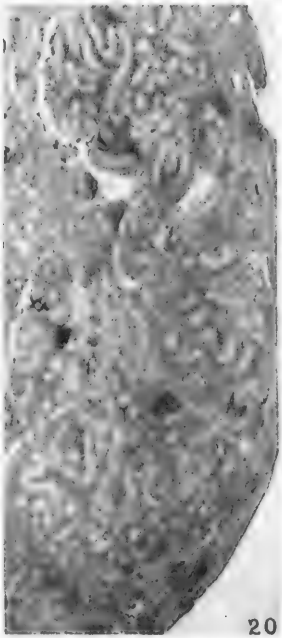

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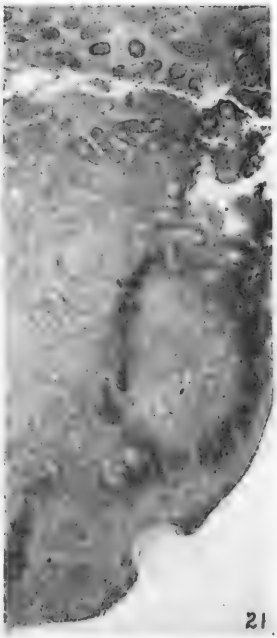

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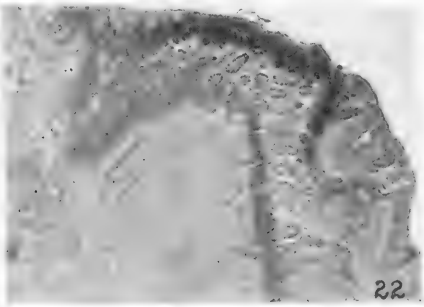
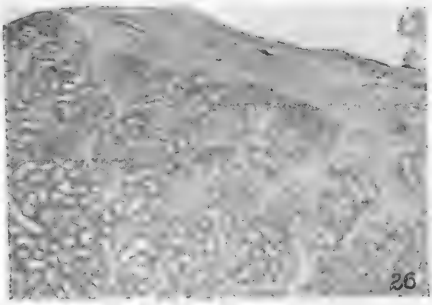
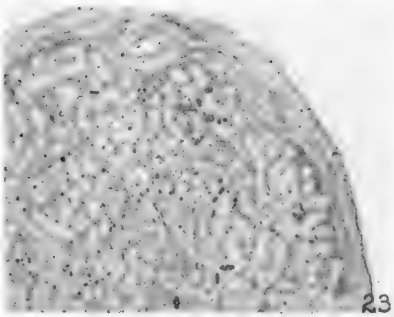
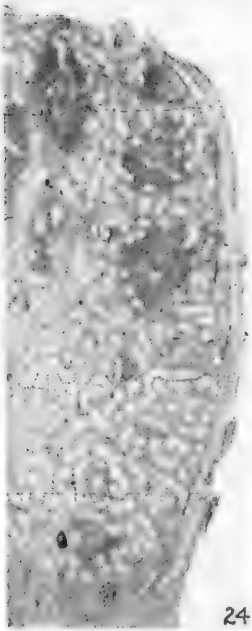
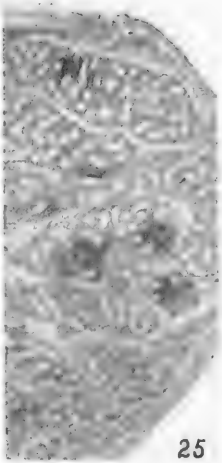
(Noguchi: Cultivation of Vaccine Virus.)

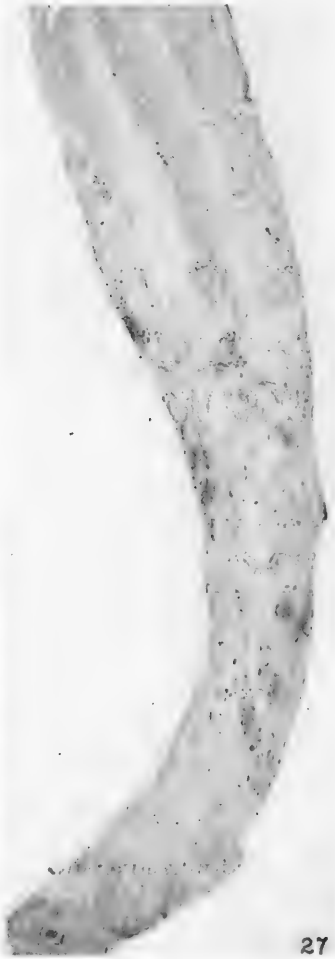




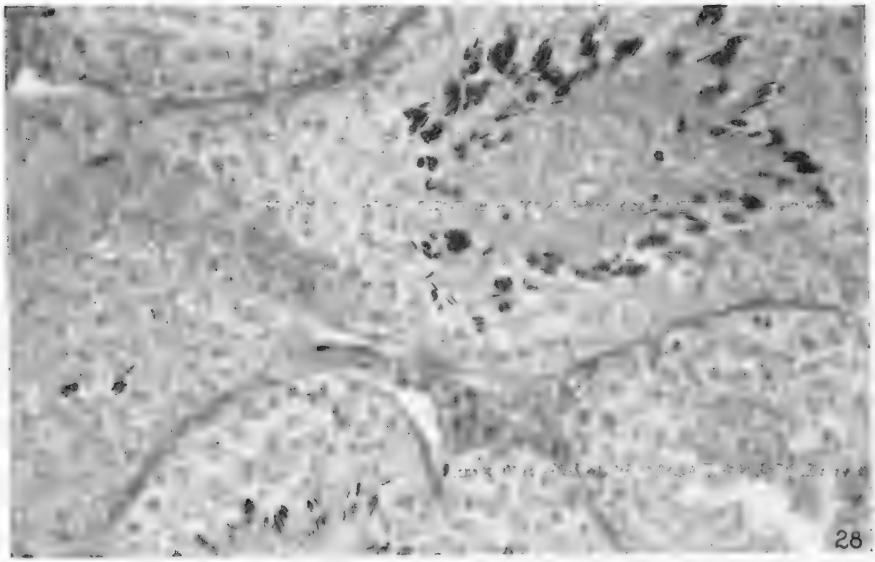


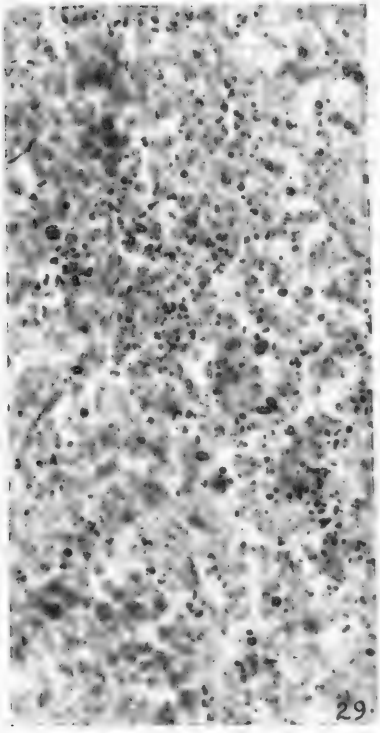


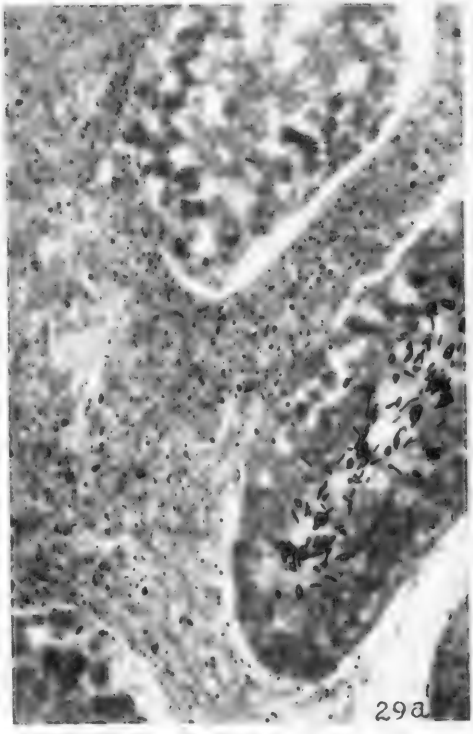




(Noguchi: Cultivation of Vaccine Virus.)







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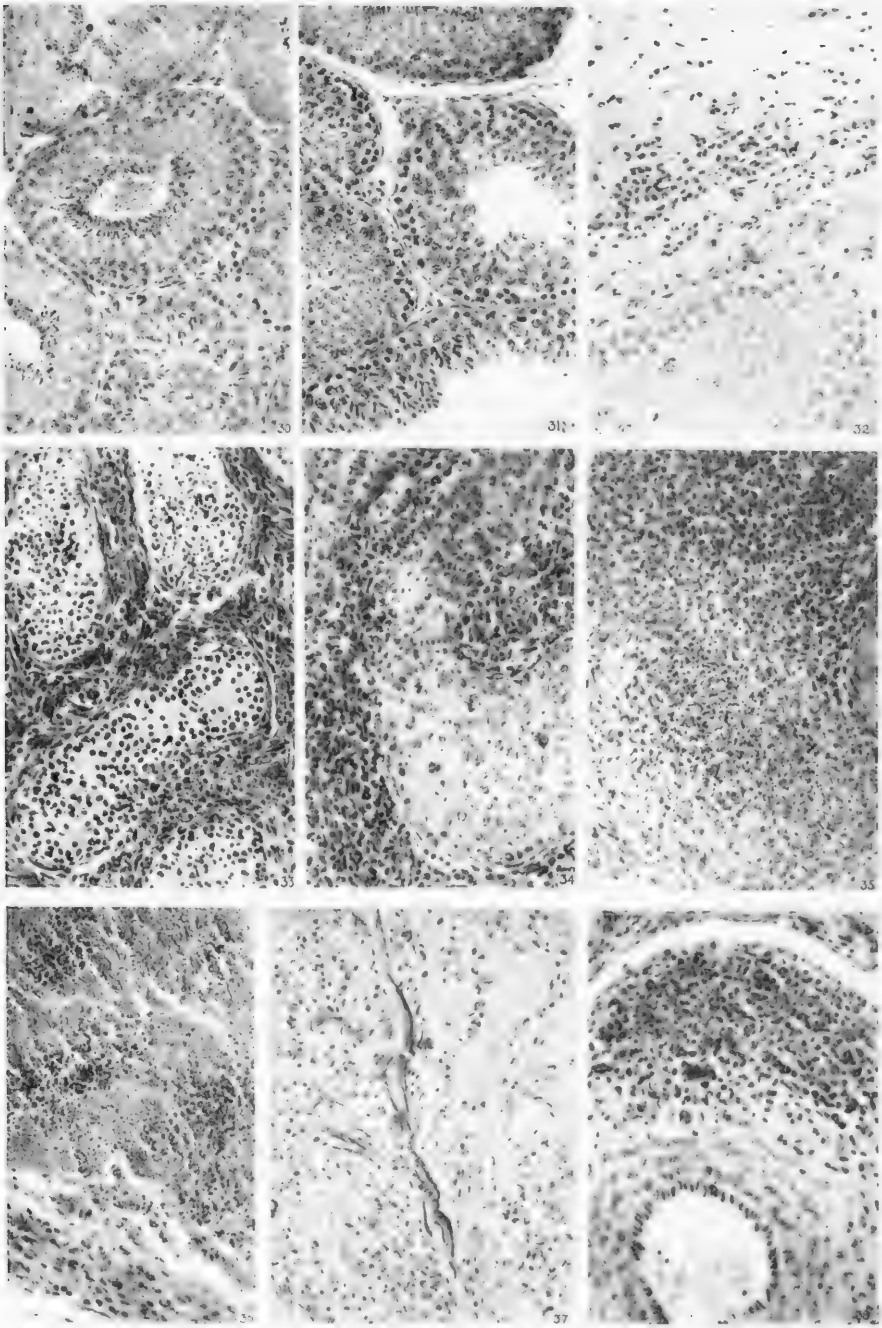

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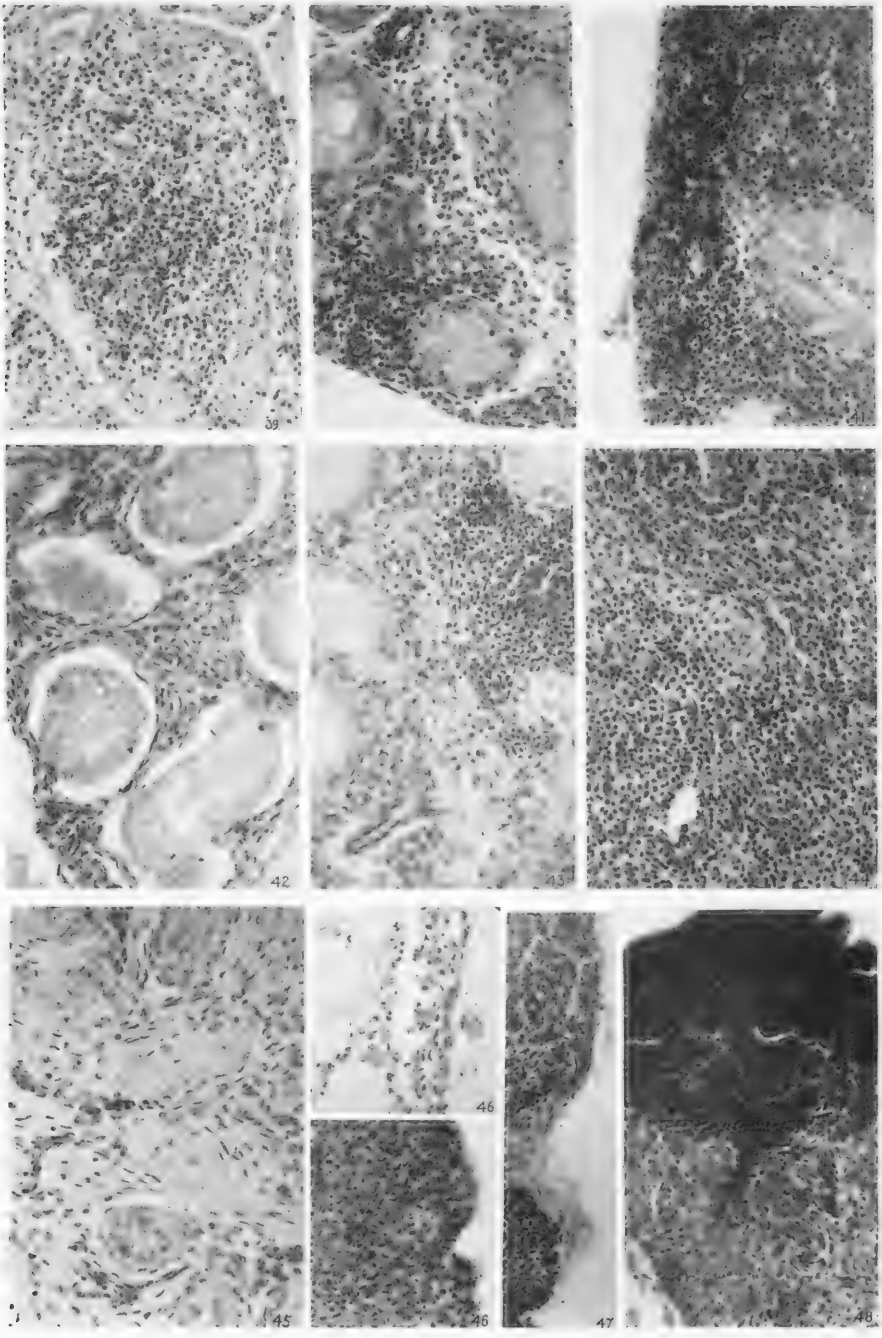

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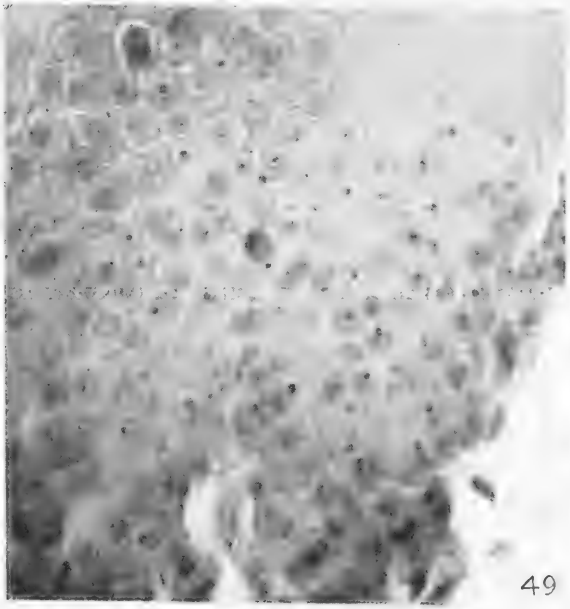

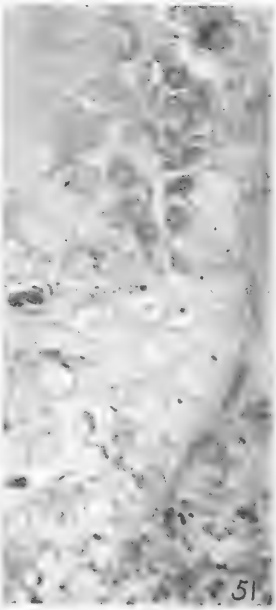

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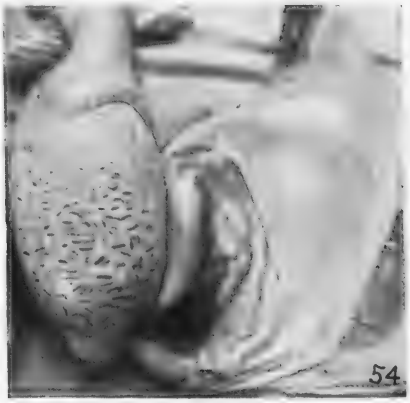

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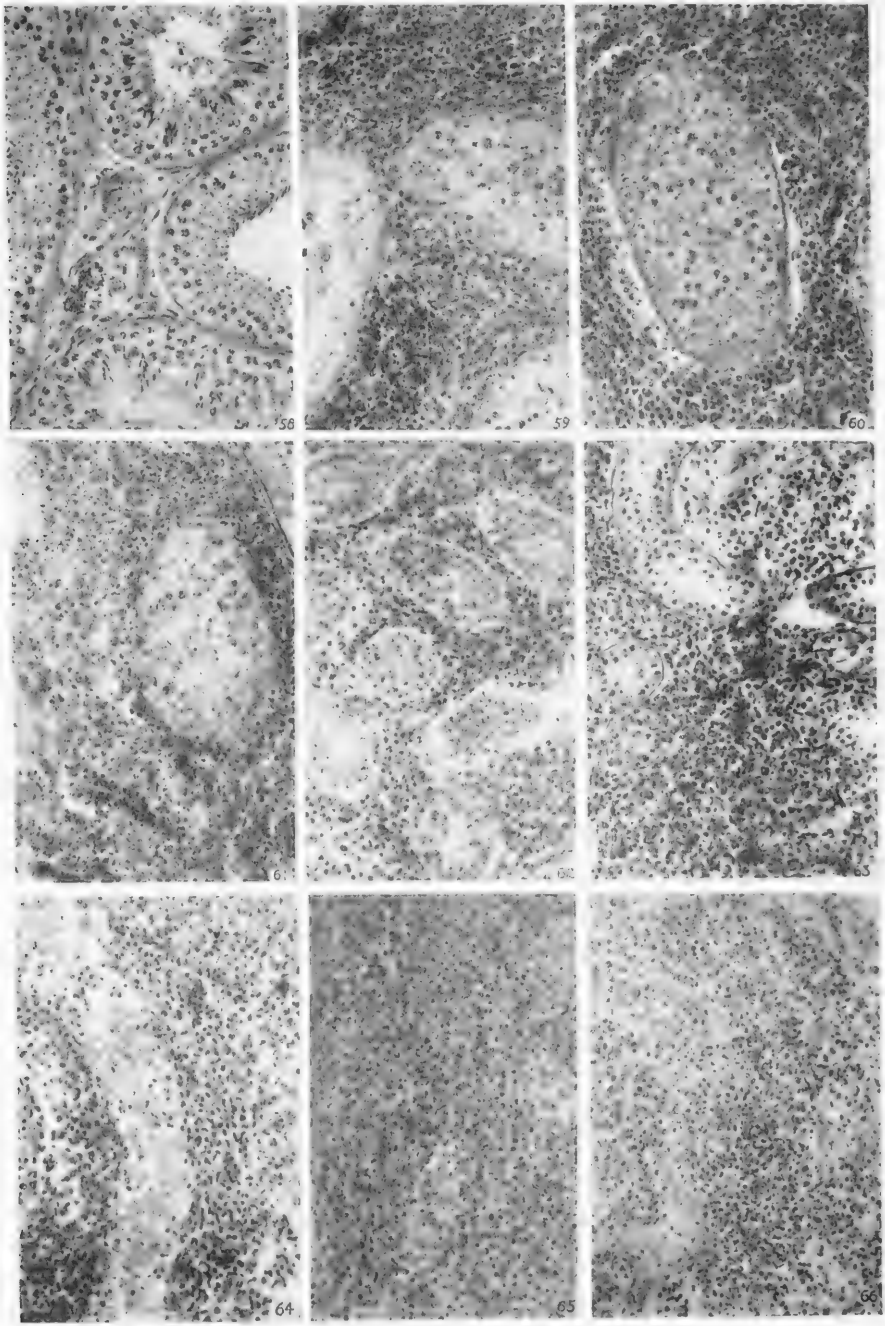

(Noguchi: Cultivation of Vaccine Virus.)

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(Noguchi: Cultivation of Vaccine Virus.)

(Noguchi: Cultivation of Vaccine Virus.)

THE TRANSPLANTATION OF TUMORS TO FOREIGN SPECIES.*

By CASIMIR FUNK, D.Sc.

PLATE 51.

It is known that when a mouse tumor is implanted into rats growth goes on for seven to ten days, but then resorption takes place. The failure of tumors to grow in animals of foreign species seems to be a general rule to which few exceptions are known.[1] Ehrlich explains the temporary growth of mouse tumors in rats on the theory, the so called athreptic theory, that the foreign organism is lacking in specific foods, which exist only in animals of the sort from which the tumor came.

The recent work on the influence of diet on tumor growth[2] has shown the importance of this hitherto undervalued factor. It seems advisable to investigate the influence of feeding to animals in which a tumor of a foreign species is growing, the tissue of that species, more especially tumor tissue itself. In this way the substance which the tumor needs might be supplied. Some time ago I tried to render rats more susceptible to mouse tumor by injections of alcoholic extracts of mouse tumor, but though some success was obtained the study had to be abandoned as the injections proved toxic.

The experiments here to be reported were arranged as follows: The tumor chosen was Ehrlich's mouse chondroma, which "takes" in a very high percentage of the mice to which it is transplanted. Before the experiments were started, many mice were inoculated with the growth to supply the tumor material used for feeding. Then a

* Received for publication, February 15, 1915.

[1] Among the reported cases of successful transplantation of human tumors to animals, or of animal tumors to other species, few will stand criticism. In very few cases indeed (Dagonet-Mauclaire, Carl Lewin) were the growths successfully reinoculated.

[2] Sweet, J. E., Corson-White, E. P., and Saxon, G. J., *Jour. Biol. Chem.*, 1913, xv, 181. Funk, C., *Lancet*, 1914, i, 98. Rous, P., *Jour. Exper. Med.*, 1914, xx, 433.

large number of rats were inoculated,—also with the chondroma,—and these were divided into two batches, one batch being fed on ordinary food, the other on ordinary food with the twice daily addition of freshly excised chondroma from the mice. The rats ate the tumor material with avidity.

The results were striking. In rats fed the tissue of the chondroma, this growth succeeded on inoculation much better than in those receiving ordinary diet.

Experiment I. First Generation.—Two batches, each of thirty young rats, were employed. One batch was fed, besides ordinary food, the mouse chondroma twice each day. After a week of the feeding both batches were inoculated with material of the mouse chondroma. The same tumor was used for all the inoculations. In the controls fed on ordinary food but one small tumor appeared. This was noticed after ten days and was completely absorbed after fifteen days. Of the rats fed the chondroma, six developed tumors, and in one instance the growth after sixteen days was about 12 by 18 mm. in size. It was disc-shaped and only 3 to 4 mm. thick. This tumor was examined microscopically and showed the histological characters of the original tumor (figure 1), but in distinction from the latter, which was soft and hemorrhagic, it was firm, and consisted, as the drawing shows, practically entirely of the chondroma capsules with very little homogeneous, intracellular substance (figure 2). Bits of it were transplanted to twenty rats and six mice. A chondroma developed in all of the latter, but much later than usual, showing that the transplantation into rats had in some way rendered the tumor unfit for mice. The results of the transplantation into rats is recorded below.

In another rat of this experiment the tumor reached, after five weeks, the size of a pea and was reinoculated into six mice with success in all cases.

Second Generation.—Twenty rats were inoculated with bits of the large tumor just described. All were fed the mouse chondroma. In one of the eighteen which survived seventeen days there was noted a tumor measuring 1 by 0.5 cm. Two other rats showed after twenty-four days tumors of the size of a small pea; and six had very small tumors which finally disappeared. The largest growth was examined microscopically and proved to be the chondroma. It was transplanted to eight rats and four mice,—to all of the latter successfully. The result in the rats was as follows:

Third Generation.—The eight rats inoculated with the tumor of the second generation were all fed the mouse chondroma. After eleven days one had a tumor the size of a big pea and five had smaller nodules, which finally disappeared. The largest tumor in this case was found microscopically to be a chondroma.

Further transplantations were abandoned because there was not sufficient material for inoculation.

Experiment II.—Twenty rats were inoculated with the mouse chondroma from a mouse of experiment I, which had been inoculated with a tumor from

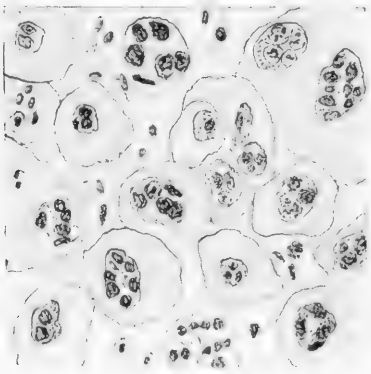

FIG. 1.

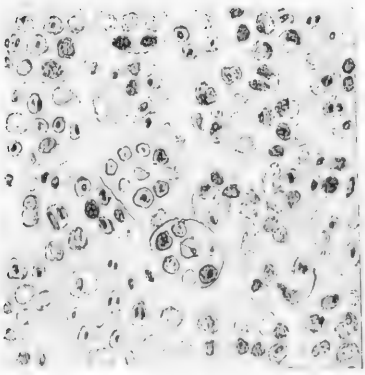

FIG. 2.

(Funk: Transplantation of Tumors to Foreign Species.)

a rat of the first generation. The animals were divided into two batches, one fed on ordinary food, the other on the same diet with the addition of tumor tissue. In the latter batch two tumors developed which still persisted after twenty-four days. One was the size of a large pea. In the controls no tumors were observed.

The experiments show that by feeding tumor tissue to animals of alien species they may be rendered more favorable to the growth of this tumor. The tumor employed, the mouse chondroma, was successfully transplanted in three successive series of rats, and kept alive for five weeks in all. But with every transplantation the tumors obtained were smaller, until the material was not sufficient for inoculation purposes. The results might have been more successful if a larger number of animals had been used, since only a very small percentage of the rats furnished tumors sufficiently large to be useful for transplantation. The method is promising enough for further experiments on other perhaps more suitable tumors and on other animals.[3]

SUMMARY.

By feeding to rats the tissue of the mouse chondroma they are rendered more suitable as hosts for the growth. Using this method rats have been inoculated successfully with the mouse tumor and it has been transferred to three successive series of these animals.

EXPLANATION OF PLATE 51.

FIG. 1. Mouse tumor (chondroma). Leitz ocular 2, objective 8.

FIG. 2. Mouse tumor (chondroma) in a rat of experiment I, generation I. Leitz ocular 2, objective 8.

[3] Following the work of Murphy (Murphy, Jas. B., *Jour. Exper. Med.*, 1913, xvii, 482) on heterogeneous transplantation in embryos, Bashford has shown that newly born animals are favorable hosts for the tumors of other species (Bashford, E. F., *Twelfth Annual Report of the Imperial Cancer Research Fund*, 1913–14, 9). The animals used in the present experiments were one-half to three-quarters grown.

THE EFFECT OF ARSENIC COMPOUNDS ON THE ROUS CHICKEN SARCOMA.*

By CASIMIR FUNK, D.Sc.

The chemotherapy of cancer, a subject much studied of late, has been investigated up to the present with rat and mouse tumors only. The results thus far obtained are contradictory, perhaps for the reason that in rats and mice a spontaneous disappearance of tumors is not infrequent, while, furthermore, diet and environment markedly influence the rate of tumor growth.

The author decided to perform some chemotherapeutic experiments with the Rous spindle celled chicken sarcoma, because, in his hands at least, this growth has never undergone spontaneous retrogression. Various arsenic compounds were selected for the test, since arsenic has shown itself to be useful in the treatment of cutaneous sarcomas. Moreover, arsenic is of use in certain infections; and there is evidence for the view that the chicken sarcoma is of an infectious nature.[1] For such reasons a curative action of arsenic seemed at least possible.

For the experiments chickens four to six weeks old of the Plymouth Rock variety were used, and the tumor was implanted in the pectoral muscle of one side. The arsenic compounds employed were arsenic and arsenious acids, cacodylic acid, atoxyl, and neosalvarsan. Fowls, as Ehrlich[2] has shown, withstand much higher doses of arsenic than do mammals. Large doses were administered intramuscularly every second day: for the first experiment into the pectoral muscle confaining the tumor, but not into the tumor itself; for the second experiment into the thigh. A slightly beneficial effect was observed as a result of the intrapectoral injections, but the injections into the thigh had no evident result on the tumor.

* Received for publication, February 15, 1915.

[1] Rous, P., and Murphy, Jas. B., *Jour. Am. Med. Assn.*, 1912, lviii, 1938.

[2] Ehrlich, P., and Hata, S., Die experimentelle Chemotherapie der Spirillosen, Berlin, 1910.

EXPERIMENTAL.

Experiment I.—Six batches of thirteen chickens each were inoculated with the Rous sarcoma. Three days after the inoculation the treatment was begun and the injections were repeated every second day, the doses being as follows:

Arsenic acid	0.1 mg.
Arsenious acid	0.1 mg.
Cacodylic acid	20 mg.
Atoxyl	10 mg.
Neosalvarsan	50 mg.

The injections were made into the pectoral muscle containing the tumor. The results of this experiment were as follows:

Of the control fowls none lived 18 days after inoculation.
Of those treated with arsenic acid 2 lived 18 days after inoculation.
Of those treated with arsenious acid 6 lived 18 days after inoculation.
Of those treated with cacodylic acid 2 lived 18 days after inoculation.
Of those treated with atoxyl 3 lived 18 days after inoculation.
Of those treated with neosalvarsan 2 lived 18 days after inoculation.

In this experiment the injection of arsenic compounds seemed to have a slight inhibiting effect on the tumor growth, especially in the case of arsenious acid, but in no case was a cure effected.

Experiment II.—In this experiment the tumor was inoculated as usual into the pectoral muscle and the arsenic compounds were injected into the muscle of the leg. The treatment was begun two days before the inoculation of the tumors and was repeated every second day.

Controls.	No. inoculated.	Survived 20 days.	Survived 27 days.
Controls	27	6	2
Arsenic acid	29	7	4
Arsenious acid	29	7	0
Cacodylic acid	29	9	2
Atoxyl	29	7	0
Neosalvarsan	30	5	1

The rapidity of growth of the tumors was the same in the treated fowls as in the controls, but in the former the tumor ulcerated more quickly.

SUMMARY.

Arsenic and arsenious acids, cacodylic acid, atoxyl, and neosalvarsan fail to influence markedly the growth of the Rous chicken sarcoma.

ANTIBODY FORMATION AGAINST TREPONEMA PALLIDUM—AGGLUTINATION.*

By HANS ZINSSER, M.D., and JOSEPH GARDNER HOPKINS, M.D.

(*From the Department of Bacteriology of the College of Physicians and Surgeons, Columbia University, New York.*)

Plates 52 to 54.

Although active immunization in syphilis has been extensively attempted by many workers, the results seem to show generally unsuccessful experiments. It is the opinion of Neisser and others that true immunity in syphilis has not so far been demonstrated and possibly does not exist. It seems that a human being is immune to reinoculation only while still diseased, but that susceptibility is again established after clinical recovery. As regards animals experimentally inoculated these matters are still uncertain, because of the unavoidable technical difficulties; for in none of the animals easily and cheaply accessible to most laboratory workers can syphilis be produced in anything like the generalized or prolonged course observed in human beings.

It has been the aim of many workers to attempt the demonstration of antibodies in the circulation of animals experimentally inoculated or treated with extracts of either syphilitic tissues or treponema culture material. Before the cultivation of *Treponema pallidum* by Noguchi's methods had made possible extensive experimentation with pure cultures, many investigators attempted active immunization of animals with tissues rich in treponemata, but with entirely unsatisfactory results.

Neisser[1] and Bruck summarize their opinion as follows: "It is plain, therefore, that parasiticidal antibodies do not occur in the course of syphilis, and that we may at most count only upon the occurrence of specific complement-fixing and agglutinating substances, although the occurrence of such antibodies can be determined with accuracy only when we shall be able to work with pure cultures of treponemata."

* Received for publication, February 20, 1915.

[1] Neisser, A., *Arb. a. d. k. Gsndhtsamte.*, 1911, xxxvii, 187, 206.

In a later summary Bruck[2] states that complement fixation in syphilis can be looked upon as only in part depending upon a specific antibody reaction. He states also that Fornet and Schereschewski's demonstration of precipitins in syphilitic sera cannot be confirmed, and that the formation of true agglutinins could not be determined by Uhlenhuth and Mulzer, who treated rabbits, goats, and monkeys with materials containing considerable amounts of treponemata. He adds, however, that this question will probably remain unsettled until the work can be repeated with materials richer in microörganisms.

Noguchi,[3] in April, 1912, published a paper on the fixation of complement when aqueous extracts of syphilis cultures were used as antigen, and the sera of patients with syphilis and of experimentally inoculated rabbits were used. He found positive fixation in the majority of the cases of secondary, tertiary, and hereditary syphilis, also in one of five syphilitic rabbits, using as antigen the emulsion from syphilitic testicles or from pure cultures. The validity of the reactions obtained with syphilitic testicles he regarded as doubtful, since emulsions from normal testicles gave, with some of the sera, equally strong reactions.

Craig and Nichols,[4] using alcoholic extracts of pure cultures of the syphilis organism and some other spirochete, found occasional fixations in syphilitic cases, but these were weaker than similar fixations obtained with the ordinary lipoid tissue extracts used in the common Wassermann technique.

Kolmer, Williams, and Laubaugh,[5] working both with aqueous and alcoholic extracts of concentrated pure cultures, found that such antigens gave positive fixation reactions with the serum of a considerable number of human syphilitic cases, and strong reactions with the serum of rabbits treated with treponema cultures.

Kolmer[6] also observed that treponemata from cultures in horse serum broth were agglutinated by the sera of rabbits which had received injections of such cultures either intravenously or into the testicle.

These later researches seem to indicate definitely that circulating antibodies may be formed in the course both of spontaneous syphilis of human beings and in animals treated with cultures of the treponema. Noguchi in summing up the problem in his paper concludes that his experiments seem to show that the ordinary Wassermann reaction is not determined by specific antibodies; that, however, the fixation produced with the culture pallida antigen seems to signify the presence of the true antibodies. He remarks upon the peculiar fact that the concentration of antibodies in syphilitic patients as determined by the pallida antigen seems to be surprisingly slight when compared with antibody formation in many other infectious diseases.

[2] Bruck, C., in Kolle, W., and von Wassermann, A., Handbuch der pathogenen Mikroorganismen, 2d edition, Jena, 1913, vii, 1060.

[3] Noguchi, H., *Jour. Am. Med. Assn.*, 1912, lviii, 1163.

[4] Craig, C. F., and Nichols, H. J., *Jour. Exper. Med.*, 1912, xvi, 336.

[5] Kolmer, J. A., Williams, W. W., and Laubaugh, E. E., *Jour. Med. Research*, 1913, xxviii, 345.

[6] Kolmer, J. A., *Jour. Exper. Med.*, 1913, xviii, 18.

That specific circulating antibodies for *Treponema pallidum* may be formed in syphilitic human beings and in experimentally inoculated animals has thus been rendered likely and has to some extent found support in experimental observation.

The observations which we desire to report in the present communication deal entirely with the appearance of specific agglutinating antibodies in the sera of rabbits intravenously treated with emulsions in salt solution of *Treponema pallidum,* killed by heating to 56° C. for thirty minutes. This temperature was chosen after it had been ascertained by Dr. Hopkins (in experiments as yet unpublished) that the thermal death point of these microörganisms lay between 50° and 55° C.

We have been studying antibody formation in syphilitic infection for some time, but our work could not be carried out on a sound experimental basis until we had developed a technique by which we could obtain larger quantities of treponema material which could be centrifugalized and washed free of culture fluid in the same way as this is done in similar experiments with bacteria. These methods have been in essence described in a previous paper.[7]

In all our antibody work in which the serum of treated rabbits was used, we took care to treat animals with treponema material that had been grown in sheep serum mixtures with salt solution or broth, whereas the treponemata used in the final reactions were grown on human ascitic fluid media. Such a technique was used in order to avoid the possibility of false reactions which might have been caused by the production of protein antibodies by antigen injected into the animals with remnants of the culture fluids. This is a precaution which we think should be taken in all experiments with this microorganism.

The material for agglutination was obtained by decanting the culture fluid from 250 cubic centimeter flasks in which the treponemata had been grown for from four to six weeks. Relatively slow centrifugation was first carried out for a short time to throw down coarse particles. After this, rapid and prolonged centrifugation yielded a sediment which when suspended in salt solution gave an evenly turbid emulsion very rich in treponemata. It should be noted

[7] Zinsser, H., Hopkins, J. G., and Gilbert, R., *Jour. Exper. Med.*, 1915, xxi, 213.

for the benefit of others undertaking these experiments that it is often difficult to free the treponema emulsion entirely from minute particles of precipitate likely to occur in old ascitic fluid mixtures. Such a precipitate often adds considerable turbidity to the final emulsion and may result in disturbing irregularities of reaction. In many of our experiments it will be noticed that ± is used, and this often signifies very slight flake formation in the tubes when such an unclean suspension was used. The reactions are as sharp and distinct as similar reactions on the agglutination of bacteria when the emulsions are unmixed with such precipitate. We have found it of advantage to discard material in which such a disturbing precipitate was present to any extent. When, as in a very few experiments, spontaneous agglutination occurred in salt solution this was of extremely slight degree only and of such a character that it was easily and sharply differentiable from that occurring in the positive tubes.

EXPERIMENT I.

Suspension Strain A (Macroscopic).

Serum of Rabbit 609 (Intravenously Treated with Five Injections of Suspension A Ranging from 1 to 5 Cubic Centimeters).

Each Tube Contained 0.5 of a Cubic Centimeter of Serum Dilution and 0.25 of a Cubic Centimeter of Suspension.

Concentration.	Serum 609. First bleeding.		Serum 609. Second bleeding.		Normal rabbit serum.	
	2 hrs.	15 hrs.	2 hrs.	15 hrs.	2 hrs.	15 hrs.
Undiluted	+++	+++	+++	+++	+++	+++
1 : 2	+++	+++	+++	+++	+++	+++
1 : 5	+++	+++	+++	+++	+++	+++
1 : 10	+++	+++	+++	+++	+++	+++
1 : 20	+++	+++	+++	+++	±	±
1 : 50	+++	+++	+++	+++	0	0
1 : 100	+++	+++	+++	+++	0	0
1 : 200	+++	+++	+++	+++	0	0
1 : 500	++	+++	+++	+++	0	0
1 : 1,000	±	0	Not set up		0	0
1 : 2,000	0	0			0	0
1 : 4,000	0	0			0	0
Salt solution (control)	0	0	0	0	0	0

EXPERIMENT II.

Suspension Strain A (Macroscopic).

Serum of Rabbit 611 (Intravenously Treated with Six Injections of Suspension A Ranging from 1 to 4 Cubic Centimeters).

Each Tube Contained 0.5 of a Cubic Centimeter of Serum Dilution plus 0.25 of a Cubic Centimeter of Treponema Suspension.

Concentration.	Serum 611.		Normal rabbit serum.	
	2 hrs.	15 hrs.	2 hrs.	15 hrs.
Undiluted	+++	+++	+++	+++
1 : 2	+++	+++	+++	+++
1 : 5	+++	+++	+++	+++
1 : 10	+++	+++	+++	+++
1 : 20	+++	+++	±	±
1 : 50	+++	+++	0	0
1 : 100	+++	+++	0	0
1 : 200	+++	+++	0	0
1 : 500	+++	+++	0	0
1 : 1,000	+++	+++	0	0
1 : 2,000	±	+++	0	0
1 : 4,000	0	±	0	0
Salt solution (control)	0	0	0	0

Since it seemed important to establish, if possible, the ordinary limits of the agglutinating properties of normal rabbit serum, we carried out the following experiment with a larger number of normal rabbit sera and a suspension of strain A. For comparison, rabbit sera 609 (five injections), 610 (five injections), and 619 (five injections) were set up with the same suspension.

EXPERIMENT III.

Concentration.	Serum 609.	Serum 610.	Serum 619.	Normal 1.	Normal 2.	Normal 3.	Normal 4.	Normal 5.	Normal 6.	Normal 7.
Undiluted	+++	+++	+++	++	++	+++	++	++	+++	++
1 : 10	+++	+++	+++	±	±	+	±	±	±	±
1 : 20	+++	+++	+++	±	±	+	±	±	±	±
1 : 50	+++	+++	+++	±	±	+	±	±	+	±
1 : 100	+++	+++	+++	±	±	+	±	±	+	±
1 : 200	+++	+++	+++	±	±	+	±	±	+	±
1 : 500	+++	+	+++	±	±	+	±	±	+++	±
1 : 1,000	+	+	+++	±	±	+	±	±	+	±
1 : 2,000	+	+	+++	±	±	+	±	±	+	±
1 : 4,000	+	+	±	±	±	+	±	±	+	±
Salt solution (control)	±	±	±	±	±	+	±	±	±	±

In interpreting this experiment it is necessary to note that the ±, or very slight flocculation, which occurred throughout in the normal

sera, occurred also in the salt solution controls. It must, therefore, be looked upon as spontaneous clumping, in our opinion due, in part at least, to the fact that the emulsion of treponemata on this day contained not inconsiderable amounts of the precipitate granules mentioned above. Moreover, this flocculation in the normal sera and in the salt solution was slow and in character recognizably different from that appearing in the tubes with immune serum, in that in the last named the flakes formed rapidly and settled out in a short time.

The irregularity in normal serum 6 we cannot explain, but feel that in spite of it our results are sufficiently convincing.

Since at this time we happened to possess good material from a mass culture of one of the strains which Dr. Noguchi had kindly sent us, his No. 9, our Noguchi I, we carried out an experiment to determine whether the serum of a rabbit treated with our strain A would agglutinate Noguchi I.

EXPERIMENT IV.

Suspension Strain Noguchi I. Serum of Rabbit 609.

Each Tube Contained 0.5 of a Cubic Centimeter of Serum Dilution and 0.25 of a Cubic Centimeter of Spirochete Suspension.

Concentration.	Serum 609. First bleeding.		Serum 609. Second bleeding.		Normal rabbit serum.	
	2 hrs.	15 hrs.	2 hrs.	15 hrs.	2 hrs.	15 hrs.
Undiluted	+++	+++	±	+++	0	++
1 : 2	+++	+++	++	+++	0	+
1 : 5	+	+++	++	+++	Not set up	
1 : 10	±	+++	0	+++	0	±
1 : 20	0	+++	0	+++		
1 : 50	+++	+++	0	+++		
1 : 100	+++	+++	±	+++	Not set up	
1 : 200	+++	+++	++	+++		
1 : 500	+++	+++	+	+++		
Salt solution (control)	0	0	0	0	0	0

DISCUSSION.

The protocols which are reported above are based entirely upon experiments done with macroscopic agglutination carried out in small test-tubes. The sharpness with which these can be read is apparent from figure 1. We have also carried out a number of microscopic agglutinations in which small drops of the mixtures of serum

dilution and treponema emulsion were placed on slides, covered with cover-slips, and rimmed with vaselin. In preparations so made it was very easy to follow the agglutination in a way exactly similar to that habitual in the usual Widal reaction in typhoid fever. The clumping was sharp and distinct, and false clumping, that is, that which would depend upon the presence of disturbing precipitate in the preparations, could easily be ruled out by this method, although our microscopic reactions thus far done were set up with very clean treponema preparations and were interpreted with absolutely no difficulties, as, we think, will be apparent from the photographs attached to this paper (figures 2 to 5).

The results we have obtained, of course, obviously suggest the possibility of working out an agglutination reaction with emulsions of *Treponema pallidum* prepared by our technique, and human sera, with possibly some diagnostic value.

We are especially encouraged in the hope, since Zabolotny and Maslakowetz[8] have observed clumping of the microörganisms in drops of exudate taken directly from primary lesions. In order to obtain some light upon this we immediately carried out a number of microscopic and macroscopic agglutination reactions with sera taken from a series of cases, some of them showing positive and some of them negative Wassermann reactions. It was revealed that many human sera used in concentration and in dilutions as high as 1 to 10 will agglutinate strain A. It will be necessary to examine a large series of normal and syphilitic sera, carefully controlled by clinical observation.

The last experiment we have reported also suggests the possibility that by agglutination it may be possible to determine that a given treponema belongs to the species pallidum. However, it will be necessary first to ascertain whether sera prepared by injections of pallidum may not also agglutinate spirochetes of other species.

SUMMARY.

It has been shown by our experiments that the serum of rabbits treated with emulsions of *Treponema pallidum* contains agglutinating substances.

[8] Zabolotny, D., and Maslakowetz, *Centralbl. f. Bakteriol., 1te Abt., Orig.*, 1907, xliv, 532.

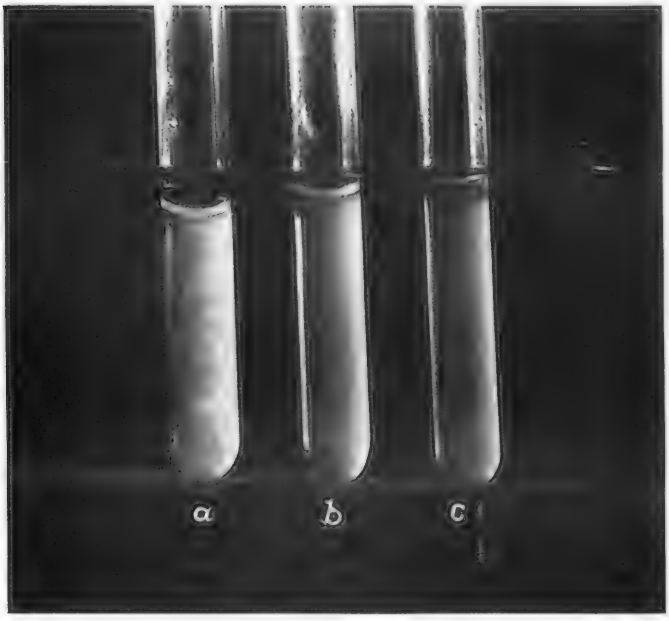

FIG. 1.

(Zinsser and Hopkins: Antibody Formation.)

FIG. 2.

FIG. 3.

(Zinsser and Hopkins: Antibody Formation.)

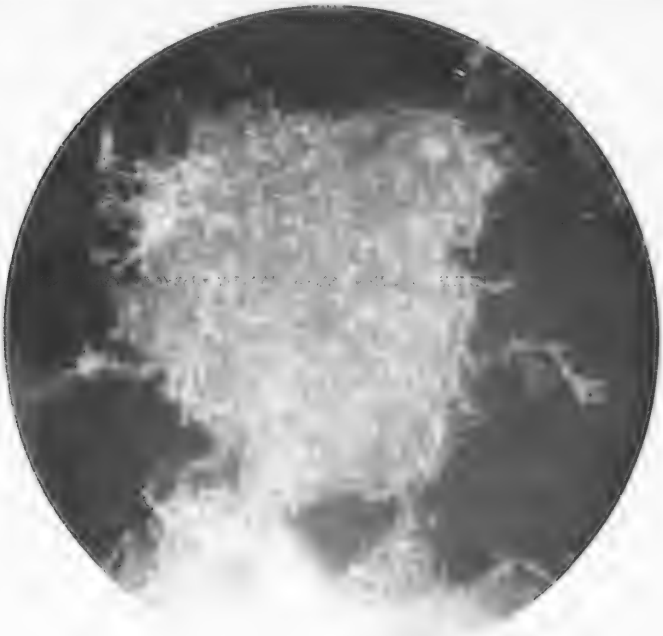

FIG. 4.

FIG. 5.

(Zinsser and Hopkins: Antibody Formation.)

Normal rabbit serum also possesses agglutinating power for this organism, but, as in the case of normal bacterial agglutinins, to an extent very much inferior to that possessed by the sera of immunized animals. Normal human sera will agglutinate similar pallidum emulsions, as will the sera of certain syphilitic patients with positive Wassermann reactions. Whether or not there is a quantitative difference of diagnostic value between the sera of normal human beings and those of syphilitics remains to be seen.

The sera of rabbits immunized with strain A agglutinate Noguchi's strain 9 in dilutions as high as 1 to 500.

We regard as the most important result of these experiments the demonstration of definite antibodies in the circulation of animals treated with dead emulsions of *Treponema pallidum*. Since it is our belief[9] that the agglutinating effect is due to an antibody essentially the same as that which produces bactericidal, precipitating, and opsonic effects, *i. e.*, that there is probably one type of antibody only, we believe that the demonstration of agglutinins establishes the fact that in syphilis as in bacterial diseases the host responds by the formation of antibodies or sensitizers specific for the treponema.

Spirocheticidal experiments with these sera, both *in vitro* and *in vivo*, are in progress.

EXPLANATION OF PLATES.[10]

Plate 52.

Fig. 1. Microscopic agglutination. A, immune serum 1 : 50. B, normal serum 1 : 50. C, salt solution control.

Plate 53.

Fig. 2. Microscopic appearance of the salt solution control.

Fig. 3. Microscopic appearance of the preparation with normal rabbit serum 1 : 50.

Plate 54.

Fig. 4. Microscopic appearance of the preparation containing immune serum 1 : 50.

Fig. 5. Microscopic appearance of the preparation containing immune serum 1 : 2,000.

[9] Zinsser, H., *Jour. Exper. Med.*, 1913, xviii, 219.

[10] We are indebted to Dr. John E. McWhorter for the photomicrographs reproduced in figures 2 to 5.

ADULT TERTIAN MALARIAL PARASITES ATTACHED TO PERIPHERAL CORPUSCULAR MOUNDS. THE EXTRACELLULAR RELATION OF THE PARASITES TO THE RED CORPUSCLES.*

By MARY R. LAWSON, M.D.

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Plates 55 to 58.

Many of the misconceptions in regard to the morphology and biology of the malarial parasite have risen, I believe, from the fact that the majority of observers have believed the parasite to be intracellular or submerged beneath the surface of the red corpuscle, and that each parasite grew up and completed its life cycle within a single red corpuscle, the segmentation of the parasite corresponding with the final destruction of the red corpuscle.

I have shown in previous publications[1] that the malarial parasite in the course of its development destroys several red corpuscles, migrating from one to another, and that when it is not free in the blood serum, it is attached to the outer surface of the red corpuscle. As Laveran[2] describes it, the parasites are "now free in the blood, now adherent to blood corpuscles."

The Method by Which the Malarial Parasite Secures Attachment to the Surface of the Red Corpuscle.—The parasite attaches itself to the outer surface of the red corpuscle by means of pseudopodia encircling a portion of the hemoglobin substance which has been squeezed up by the parasite into a mound with a circular base and a more or less rounded apex. The pseudopodia encircle the mound

* Aided by a grant from The Rockefeller Institute for Medical Research. Received for publication, March 29, 1915.

[1] Rowley-Lawson, M., *Arch. Int. Med.*, 1912, ix, 420; *Jour. Exper. Med.*, 1913, xvii, 324; 1914, xix, 450, 523.

[2] Laveran, A., Paludism, translated by Martin, J. W., London, 1893, 12.

at its base. The corpuscular mounds may be encircled by a single pseudopodium or by two pseudopodia meeting and fusing.

I believe that the first pseudopodia thrown out arise from the cytoplasm of the parasite near the nucleus. Other (accessory) pseudopodia may be thrown out from any part of the cytoplasm, whenever it becomes necessary for the parasite to secure a firmer hold on the red corpuscle; *i. e.,* when a corpuscular mound to which it is attached becomes dehemoglobinized (decolorized), or as the parasite increases in size.

A very young parasite may find it necessary to use its entire cytoplasm in order to encircle a corpuscular mound, thus giving the appearance, when attached to a surface mound, of a very slender ring with a chromatin dot. When a corpuscular mound encircled by a young parasite is viewed from above, the characteristic ring-form parasite picture is seen.

Young Parasites Attached to Peripheral Corpuscular Mounds.— (Figures 1 to 10.) The above interpretation of the ring-form parasite is confirmed by seeing the mounds of corpuscular substance projecting at the periphery of the red corpuscles encircled by the pseudopodia of the parasites.

The encircling pseudopodia may be wholly or in part obscured by the nuclei of the attached parasites; but frequently they may be traced around the peripheral mounds (figures 1 and 10).

Peripheral corpuscular mounds encircled by young parasites are best seen when the corpuscle is newly invaded and the hemoglobin of the mound entirely or nearly intact. As a rule, the corpuscular mounds are rapidly decolorized, proceeding as the parasite digests the hemoglobin. The various stages between the unaltered deeply colored mounds and those which are entirely decolorized may be observed (figures 1 to 10). Rarely the red corpuscle is decolorized before the corpuscular mound.

The decolorized corpuscular mound has been called the "achromatic area" and "milky zone," and many observers have believed it to be an essential part of the parasite.

As additional proof of the extracellular relation of the young parasites to the red corpuscle, the nuclei or the protoplasm may often be seen to project beyond the periphery of the red corpuscle (figures 6, 7, 9, and 10).

Pigment in Connection with Young Parasites.—The secretion of pigment by the young tertian parasites seems to follow no known rule. Its first appearance, even in the parasites of a single blood smear, is variable. The decolorization of a corpuscular mound (or even of an entire red corpuscle) may be complete without the appearance of any demonstrable pigment in the young parasite attached to it (figure 6, see parasite at o), and a young parasite without pigment may be of larger growth than a parasite with pigment. And one may see two parasites apparently of equal growth, one with and one without pigment (compare parasites without pigment in figures 1, 3, and 5, with the parasite showing a pigment granule at o in figure 2). And in instances of multiple infection of corpuscular mounds by two young parasites of an apparently equal growth, only one of these parasites may show pigment (figure 7).

This irregularity as to the time when the first pigment appears in the individual young parasite may have no significance other than that certain parasites are slower than others in securing attachment to the red corpuscles, and it may be that some of the parasites are less active than others in the secretion of pigment.

While the presence of pigment in the young parasite has a positive value, its absence does not mean that the parasite has not done its share of injury to the red corpuscle.

Infection of Corpuscular Mounds by More than One Young Parasite.—Infection of corpuscular mounds by two or more parasites may be seen (figures 4, 5, x, 6, x, and 7). It has no significance other than accidental occupation. Parasites occupying the one corpuscular mound are usually of one brood and, therefore, in the same stage of development; but parasites of varying ages may also be seen occupying the one corpuscular mound (figure 6, x).

In multiple infections of corpuscular mounds the parasites proceed with the destruction of the mounds in the same way as do single parasites.

Adult Tertian Parasites Attached to Peripheral Corpuscular Mounds.—(Figures 11 to 72, and 75 to 108.) While several observers have conceded that the young parasite may be extracellular for a certain time, there has been, with but few exceptions, quite a unanimity of opinion amongst the older writers that the adult para-

site is always intracellular. I have been asked several times if it was possible to demonstrate any appreciable number of adult tertian parasites attached to peripheral corpuscular mounds. These inquiries led to an exhaustive study in search of the same.

Adult parasites are usually seen attached to surface mounds; but peripheral mounds may be overlooked or not recognized, except by the expert observer. Here and there you find adult tertian parasites attached to perfectly recognizable peripheral corpuscular mounds. I believe that adult tertian parasites attached to peripheral corpuscular mounds are only to be found in appreciable numbers in any one smear, soon after a parasitic migration when the infected red corpuscles are not badly damaged; then the peripheral mounds may show more or less of hemoglobin content. But such a condition is so transient that you may have to search for days and examine many specimens before you find what you want.

Corpuscular mounds are quickly dehemoglobinized by adult parasites (figures 50 at 0, 65, 69 to 73, and 105 to 108); I believe more quickly than by the young parasites.

If the tertian malarial parasites were attached to but one red corpuscle during their life time and did not migrate to fresh red corpuscles, I believe that it would be practically impossible to find peripheral corpuscular mounds in connection with the adult parasites.

The adult tertian parasites follow the same procedure in attaching themselves to the red corpuscles as do the young parasites. Primary and accessory pseudopodia encircle surface mounds. As a rule, the pseudopodia which encircle the peripheral corpuscular mounds arise from the cytoplasm of the parasites in the vicinity of the nuclei, as is the case with the young parasites.

The bases of some of the corpuscular mounds in connection with the adult parasites are more constricted than is usual with the mounds seen in connection with the young tertian parasites; *i. e.*, figures 12, 20, 21, and 23. This would indicate greater strength exerted by the adult parasites when encircling the corpuscular mounds.

The nuclei of the adult parasites usually hide from view the encircling pseudopodia, but occasionally the pseudopodia may be seen en-

circling the peripheral mounds (figures 11, 12, 13, 14, 16, 27, 32, 39, 52, 56, 57, 59, 61, 69, 73, 75, 76, 77, 79, 96, 97, 101, 102, and 106).

The nuclei of the adult parasite often extend beyond the periphery of the red corpuscle (figures 12 and 14, 76 and 79), which would not be the case were the parasite intracellular. And some of the adult parasites are so attached to the surface of the red corpuscle that even to the untrained eye they admit of no other interpretation than that they are attached to the outside of the red corpuscle (figures 74 and 109).

Pigment in Connection with Adult Parasites.—Adult parasites, apparently in similar stages of development, may show not only a variation in the amount of pigment secreted, but a variation in the size and shape of the granules. Various explanations have been given to account for this, but I shall not enter into a discussion of them in this paper.

Multiple Infection of Corpuscular Mounds by Adult Parasites.—More than one adult parasite may be seen to be attached to a single corpuscular mound (figures 18, 19, 44, 63, 72, 86, 87, and 100). As is the case with the young parasites, adults occupying the one mound may be in similar or varying stages of development. Their occupancy of the one mound I believe to be accidental.

I have never found any evidence of the conjugation of malarial parasites. Two parasites attached to one surface mound, with their pseudopodia encircling it superimposed, when viewed from above might give an appearance of conjugating parasites, provided one believed the parasites to be intracellular; but when one knows that they are attached to the one corpuscular mound on the surface of the red corpuscle, then their position in relation to each other is easily explained.

Mounds of hemoglobin substance projecting out at the periphery of the red corpuscles, encircled by the pseudopodia of the two adult parasites, assist in the interpretation of the surface mounds encircled by two adult parasites.

The infected red corpuscles in the pictures which I have selected to illustrate this article show no evidence of crenation or other technical injury.

SUMMARY.

1. The malarial parasite is extracellular throughout its entire life cycle; that is, when it is not free in the blood serum, it is attached to the external surface of the red corpuscle.

2. Adult parasites follow the same procedure in attaching themselves to the outer surface of the red corpuscles as do the young parasites.

3. Adult parasites are most frequently seen attached to surface corpuscular mounds.

4. Corpuscular mounds projecting at the periphery of the red corpuscles and encircled by the pseudopodia of adult parasites, are proof positive of the extracellular relation of the adult parasite to the red corpuscle.

5. Adult parasites attached to peripheral corpuscular mounds are only found in appreciable numbers when the red corpuscles are not badly damaged, so that the mounds show more or less hemoglobin content.

6. The nuclei or protoplasm of adult parasites extending beyond the periphery of the red corpuscles is additional evidence of the extracellular relation of the parasites to the red corpuscle.

EXPLANATION OF PLATES.

PLATE 55.

TERTIAN MALARIAL PARASITES ATTACHED TO PERIPHERAL CORPUSCULAR MOUNDS.

Magnification, × 1,900.

FIG. 1. A young parasite attached to a peripheral corpuscular mound. The pseudopodium may be seen encircling the mound.

FIG. 2. A young parasite attached to a peripheral corpuscular mound. A pigment granule may be seen at o.

FIG. 3. A young parasite attached to a partly decolorized corpuscular mound.

FIG. 4. Two young parasites attached to one peripheral corpuscular mound. The nucleus of one parasite partly overlies that of the other parasite, giving the appearance of one large nucleus, but a careful examination will show the presence of two parasites.

FIG. 5. A young parasite attached to a peripheral corpuscular mound and two young parasites attached to a single corpuscular mound on the surface of the red corpuscle.

FIG. 6. Two young parasites, varying somewhat in size, attached to a peripheral corpuscular mound; and at o, a young parasite attached to a completely decolorized peripheral mound.

FIG. 7. Two tiny parasites (it requires the entire cytoplasm of each parasite to encircle the mound) attached to a decolorized peripheral mound. One of the parasites shows a granule of pigment at x.

FIG. 8. A young parasite attached to a peripheral mound which is almost decolorized. A granule of pigment may be seen in connection with the parasite.

FIG. 9. A young parasite attached to a decolorized peripheral mound.

FIG. 10. A young parasite attached to an almost decolorized peripheral mound. A careful examination will show the encircling pseudopodium on the under surface of the mound.

FIG. 11. Two parasites attached to peripheral corpuscular mounds; one of the parasites is much smaller than the other.

FIGS. 12, 13, and 14. Adult parasites attached to peripheral corpuscular mounds. The pseudopodium of each parasite may be seen encircling the mound.

FIGS. 15, 16, and 17. Adult parasites attached to peripheral corpuscular mounds. Figure 15 shows the nucleus of the parasite overlying the encircling pseudopodium. In figure 16 the pseudopodium may be seen on the under surface of the mound. In figure 17 the nucleus of the parasite partly overlies the encircling pseudopodium.

FIGS. 18 and 19. Two adult parasites attached to the one peripheral corpuscular mound.

FIG. 20. An adult parasite attached to a peripheral mound. The mound is somewhat constricted at the base.

FIG. 21. An adult parasite attached to what is really a surface mound, with the periphery of the corpuscle so turned over that the surface mound presents as a peripheral mound. This mound is somewhat constricted at the base.

FIGS. 22, 23, 24, 25, and 26. Adult parasites attached to peripheral corpuscular mounds. Figure 23 shows two peripheral mounds, one of them being very much constricted at its base.

PLATE 56.

ADULT TERTIAN MALARIAL PARASITES ATTACHED TO PERIPHERAL CORPUSCULAR MOUNDS.

Magnification, × 1,900.

FIGS. 27 to 50. Adult parasites attached to peripheral mounds. Figures 29 and 33 show each parasite attached to two peripheral mounds. Figure 50 shows an entirely decolorized mound at o. Figure 44 shows two parasites attached to one peripheral mound.

PLATE 57.

ADULT TERTIAN MALARIAL PARASITES ATTACHED TO THE EXTERNAL SURFACE OF THE RED CORPUSCLES.

Magnification, × 1,900.

FIGS. 51 to 59. Adult parasites attached to peripheral mounds.

FIG. 60. An adult parasite attached to what is really a surface mound. The periphery of the corpuscle is turned over so that the surface mound presents as a peripheral mound.

FIGS. 61 and 62. Adult parasites attached to partially decolorized peripheral mounds.

FIG. 63. Three parasites attached to one peripheral mound. The mound is much decolorized.

FIG. 64. An adult parasite attached to a partially decolorized peripheral mound.

FIG. 65. An adult parasite attached to a decolorized corpuscular mound.

FIG. 66. An adult parasite attached to a peripheral mound. The mound shows a partial decolorization.

FIGS. 67 and 68. Adult parasites attached to peripheral corpuscular mounds which have been partly dehemoglobinized by the action of the parasites.

FIGS. 69, 70, and 71. Adult parasites attached to completely decolorized corpuscular mounds.

FIG. 72. Two adult parasites attached to a completely decolorized corpuscular mound.

FIG. 73. An adult parasite attached to a decolorized corpuscular mound.

FIG. 74. An adult parasite attached to the surface of the red corpuscle in such a manner that even the most skeptical observer would not think it to be other than extracellular.

PLATE 58.

COLORED PHOTOGRAPHS OF THE PARASITES SHOWN IN PLATES 55, 56, AND 57.

Magnification, × 1,900.

FIG. 75. (Corresponds to figure 11.) Two parasites in varying stages of development attached to peripheral corpuscular mounds.

FIGS. 76, 77, 78, and 79. (Correspond to figures 12, 13, 28, and 14.) Adult parasites attached to peripheral corpuscular mounds. The pseudopodia of the parasites in figures 76, 77, and 79 may be seen encircling the mounds.

FIG. 80. (Corresponds to figure 15.) Adult parasite attached to a peripheral mound. The nucleus of the parasite overlies the encircling pseudopodium.

FIG. 81. (Corresponds to figure 16.) An adult parasite attached to a peripheral mound. The pseudopodium may be seen encircling the mound.

FIGS. 82, 83, and 84. (Correspond to figures 17, 20, and 22.) Adult parasites attached to peripheral mounds. Figure 83 shows the base of the mound to which the parasite is attached to be slightly constricted.

FIG. 85. (Corresponds to figure 21.) An adult parasite attached to what is really a surface mound. The periphery of the red corpuscle is turned over so that the surface mound presents as a peripheral mound.

FIGS. 86 and 87. (Correspond to figures 18 and 19.) Two adult parasites attached to one corpuscular mound.

FIGS. 88, 89, and 90. (Correspond to figures 24, 25, and 47.) Adult parasites attached to peripheral corpuscular mounds.

FIG. 91. (Corresponds to figure 29.) An adult parasite attached to two peripheral corpuscular mounds.

FIGS. 92, 93, and 94. (Correspond to figures 31, 34, and 35.) Adult parasites attached to peripheral corpuscular mounds.

FIG. 95. (Corresponds to figure 23.) An adult parasite attached to two peripheral corpuscular mounds. One of the mounds is very much constricted at its base.

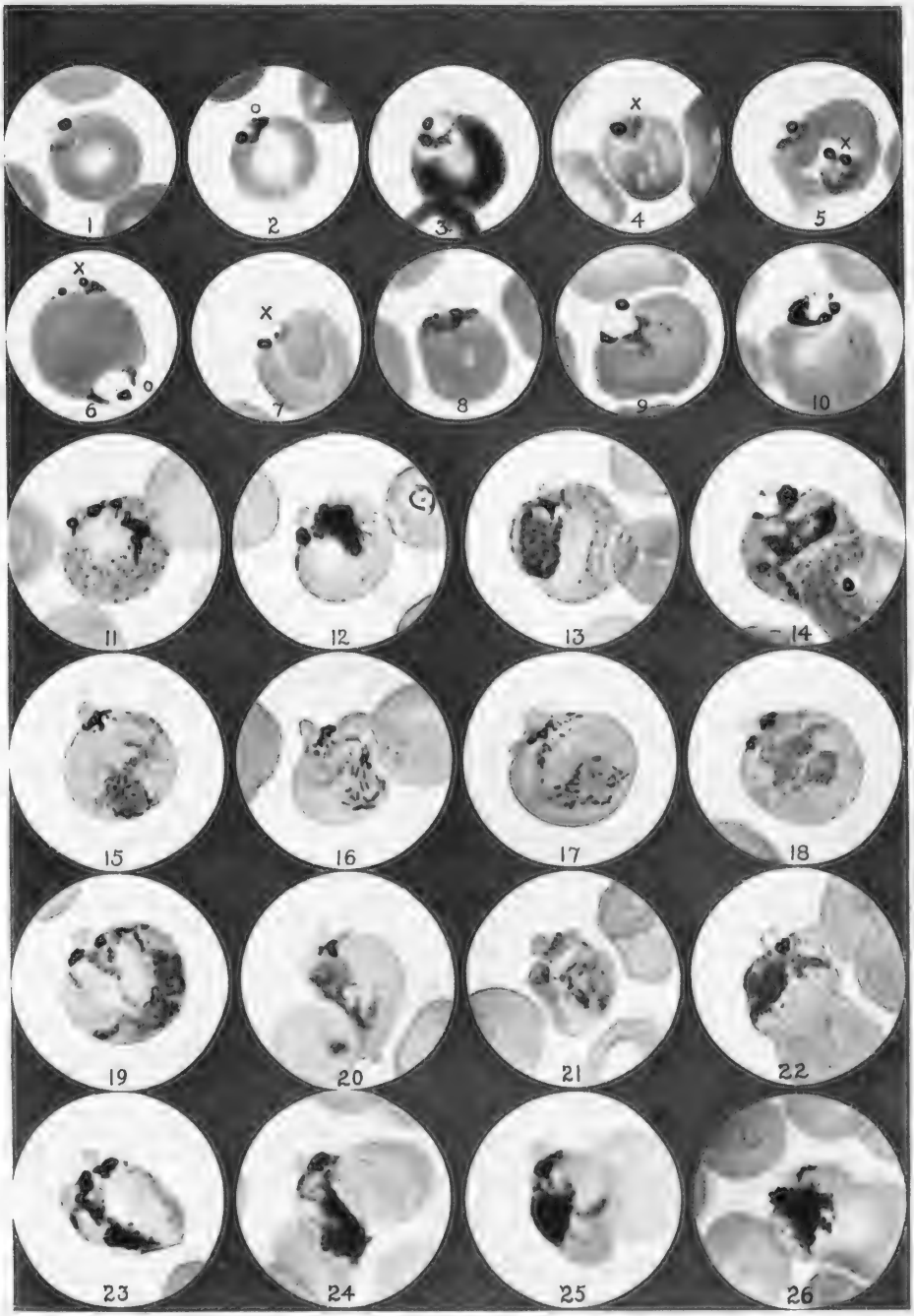
FIGS. 96, 97, 98, and 99. (Correspond to figures 39, 52, 41, and 42.) Adult parasites attached to peripheral corpuscular mounds.

FIG. 100. (Corresponds to figure 63.) Three parasites attached to one corpuscular mound.

FIGS. 101, 102, 103, and 104. (Correspond to figures 37, 61, 62, and 40.) Adult parasites attached to more or less decolorized corpuscular mounds.

FIGS. 105, 106, 107, and 108. (Correspond to figures 50, 69, 70, and 71.) Adult parasites attached to decolorized corpuscular mounds.

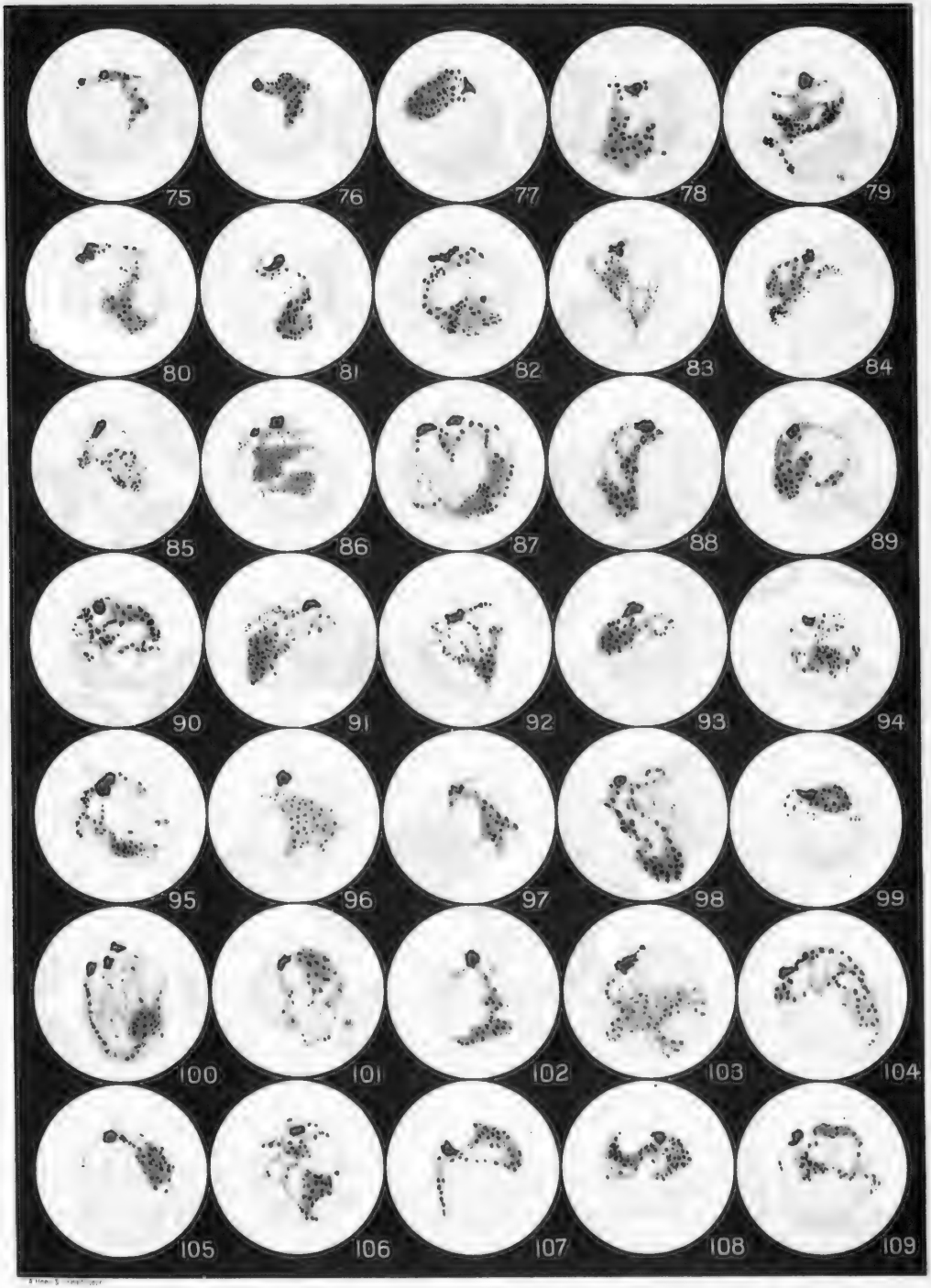
FIG. 109. (Corresponds to figure 74.) An adult parasite attached to the external surface of the red corpuscle.



(Lawson: Adult Tertian Malarial Parasites.)

(Lawson: Adult Tertian Malarial Parasites.)

(Lawson: Adult Tertian Malarial Parasites.)



(Lawson: Adult Tertian Malarial Parasites.)

THE INFLUENCE OF DIGITALIS ON THE T WAVE OF THE HUMAN ELECTROCARDIOGRAM.*

By ALFRED E. COHN, M.D., FRANCIS R. FRASER, M.D., AND ROSS A. JAMIESON, M.D.

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PLATES 59 TO 64.

The modifications in the form of the T wave of the electrocardiogram which we report in this communication were observed in the course of detailed studies on the action of digitalis in patients (1). It has been noticed frequently that the time when digitalis takes effect is a matter of uncertainty, so that in a given instance doubt is often entertained as to whether the drug itself is active, and if active, whether it is effective in producing a result. To be certain that the drug is effective, it has been considered necessary to administer it until an alteration in the rhythm of the heart occurs. In our treatment of patients we have, therefore, looked for evidences of altered heart rhythm to indicate that the heart is really digitalized. During the period of treatment, electrocardiograms were made almost daily, and often more than once a day. In examining these records we found the changes in the T wave which we describe in this report.

Other observers have published studies dealing with the effect of a number of agents, including digitalis, on the electrocardiogram. The only investigation dealing with this subject in human beings has been made by Nicolai and Simons (2). Seven ambulatory patients were given powdered digitalis, 0.3 gm. daily, for five days. In two women the treatment was not completed; in one normal man the T wave did not increase in height, but decreased instead (*"verschlechterte sich"*); and in the other four the T wave increased. They limited the taking of records to the first lead (right arm to left arm). Even if the

* A preliminary report was made to the Section on Medicine at the Seventeenth International Congress of Medicine, London, 1913, Section VI, 255–258. Received for publication, April 7, 1915.

dosage had been sufficient, and had been satisfactorily controlled, a comparison of their methods with ours would account for the difference in our results.

A number of experiments on animals have been published in which digitalis bodies, more especially strophanthin, were injected for the purpose of observing possible changes in the electrocardiogram under the influence of the drug. Straub, Selenin, Bickel and Tsividis, Bickel and Pawlow, and Rothberger and Winterberg have published such reports. In general all observers have detected an increase in size of the T wave under the influence of digitalis, like that described by Nicolai and Simons. Straub (3) described experiments in cats and rabbits, into the veins of which he injected strophanthin (Böhringer). So called therapeutic doses, the exact quantity of which is not given, caused no alterations in their electrocardiograms. When he gave toxic doses, that is to say, 0.3 mg., he obtained an increase in the elevation of the T waves in one experiment, the curve of which he reproduces. He obtained a multiplicity of abnormal ventricular complexes in the late stages of intoxication, but obviously the T waves in these forms cannot be compared with those in the control curves. In a later paper (4) he described experiments on the isolated frog heart, and showed that in the therapeutic stage the T wave increased in size, as in his mammalian experiments, while in the late toxic stages the T wave became negative. The changes in the late toxic stage in frogs are not like those in mammals, because the former do not develop abnormal ventricular complexes.

Selenin (5) found that the T wave increased in size when a digitalis body was given in therapeutic doses to dogs.

Bickel and Tsividis (6) injected digitalysatum Bürger into the ear veins of rabbits. Small and medium doses (1 c.c. per kg.) increased the size of the waves. Larger doses decreased the size of the waves, including the T wave, but they remained upward in direction.

In a later paper Bickel and Pawlow (7) injected crystalline strophanthin (Thoms) intravenously into dogs, and digistrophan, a combination of digitalis and strophanthin, into dogs and rabbits. They concluded that small doses increased the size of the T wave, whereas large doses decreased the size of all waves. They mention no instance of inversion. They found that the T wave tended to change less than the other waves. Other substances supposedly effectual on the heart, like cardiotoxin, a mixture of convallaria and caffein sodium benzoate, were quite ineffectual.

Rothberger and Winterberg (8) found that small doses of strophanthin given to dogs had no effect on the shape of the electrocardiogram. When P and T waves were small as the result of the heart's having been isolated from the central nervous system, they report that digitalis restored them to their initial size. When they gave digitalis in progressively larger quantities, aside from alterations in the P, R, and S waves, the T wave became either positive or negative. In the experiments of all these investigators the results depend on the injection of single doses of the drug, the quantities being larger than those which are employed in therapeutics. The change in the electrocardiogram usually obtained is considered to be an increase in the elevation of the T wave. Straub alone seems to have regarded the change in sign in the T wave in his experiments on frogs as unusual and important.

The shape of the T wave has also been changed by the use of other means.

Muscarin, according to Samojloff (9, 10, 11) and Boruttau (12), alters not only its form, but in frogs changes the sign of the wave from positive to negative. After the alteration has taken place, both noticed that the application of atropin restores the initial direction of the wave. Mines (13) believed that spread from the site of application to the ventricular base was responsible for Samojloff's result and that muscarin does not alter the sign of the T wave except on direct application. After alteration has taken place, atropin relieves it. But in his figures 2 and 3 the sign of T changed from negative to positive. Here the heart was perfused with muscarin so that the possibility of local action is excluded. Samojloff and Mines (14, 15) both observed that electrical stimulation alone of the vagus nerve in frogs without the use of drugs produced an inversion of the T wave.

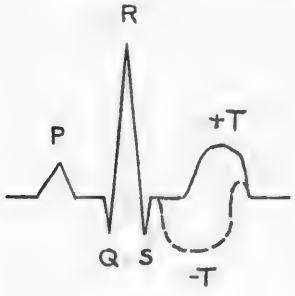
OBSERVATIONS.

In the curves of the patients under treatment with digitalis, attention was arrested by the fact that alterations in the T waves of the electrocardiograms occurred in a large number (30 times in 34 patients), and also by the fact that the change was detected before alterations in rhythm or conduction time had occurred and before symptoms referable to the gastro-intestinal tract disturbed the patients, except in five instances. The changes in the T wave consisted in a diminution in the height of the wave, and, finally, in an inversion (figure 1). This diminution in height to an isoelectric line and subsequent inversion of the wave are not the only changes which occurred. If the space from the end of R or S to the end of T is considered, additional alterations become evident. The first part of this interval, from R or S to T, is usually isoelectric or shows a slight upward slope. In place of these conditions may be substituted either an isoelectric period, if it is not isoelectric already, or a downward deflection, directly continuous with the end of R or S. The downward deflection is often carried to a point just short of where in the uninfluenced curve the T wave ends. At this time, then, the inverted wave passes above the base line to form an upward deflection, representing the terminal portion of the original wave. It is indicated in figure 1 D. Text-figure 1 shows this relation. The solid line indicates the outline of the initial curve; the dotted line, the alteration described. Samojloff (11) has drawn a similar diagram.[1] Another alteration which is sometimes seen is shown in text-figure 2. Here the downward deflection occurs, not in the

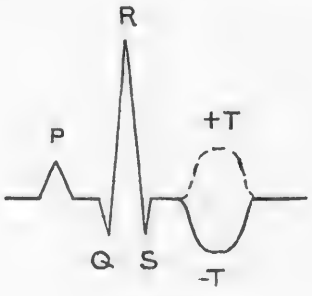
[1] Samojloff, *loc. cit.* (11), p. 481, figure 2 C.

early portion of the period, but in the later portion. Instead of a diphasic wave in which the upward deflection occupies the last portion, as in text-figure 1, it is in such instances in the first portion.

Other alterations beside those described have been seen when digitalis was administered. One was in a case of complete auriculoventricular dissociation. The observation in this patient was especially valuable because he showed, at different times, two quite differ-



TEXT-FIG. 1.



TEXT-FIG. 2.

TEXT-FIGS. 1 and 2. The unbroken line represents the electrocardiogram, and shows the P, Q, R, S, and T waves. The broken line shows the outline the T wave assumes under the influence of digitalis.

ent electrocardiograms. One indicated an origin for the ventricular beats in the left side of the heart (figure 2); the other, in the right side (figure 3). In figure 2, taken when the ventricular beats were initiated on the left side, inverted T_1[2] became deeper under the influence of digitalis, inverted T_2 moved upward, and so did diphasic T_3. When the ventricular beats were initiated on the right side (figure 3) diphasic T_1 became isoelectric, inverted T_2 turned upward, and inverted T_3 became shallow, almost isoelectric, and diphasic, while the completely restored curve resembled its control except that T_1 was a monophasic wave, replacing a diphasic wave in the initial curve. That the change was less far reaching in figure 3 than in figure 2 may have been due to the lesser amount of digitalis which had been given, 1.9 instead of 2.6 grams. These curves supply an instance of a change in the sign of T_2 and T_3 from negative to posi-

[2] T_1 represents the T wave in lead 1; T_2 in lead 2, etc.

tive under the influence of the drug, a change of sign in the reverse direction from that first described.

Still other types of change have been found. In a case, for instance, in which T_1 and T_2 were positive, and T_3 negative (figure 4), T_2 became negative, and T_3 more negative. In pathological hearts, such as these, the redistribution of muscle, resulting from hypertrophy and dilatation, doubtless determines a variety of alterations in the electrocardiogram under the influence of digitalis. We do not at the present time possess a sufficient number of instances of a given class to permit us to reduce the alterations in them to definite order. Originally it appeared to us that T_3 altered earlier than T_2. Our later observations make us hesitate to state definitely whether the T wave in one lead is influenced earlier than in the others, or whether changes occur simultaneously in all. Recent interpretations of the electrocardiogram by Einthoven, Fahr, and de Waart show that simultaneous alterations may be anticipated, and our later observations indicate that this is so.

The instances so far described relate to cases in which the mechanism of the heart beat was normal. Similar alterations have, however, also been seen when the auricles were fibrillating (figure 5) and also when they fluttered. In curves of this type, recognition of altered T waves may occasionally be difficult, especially if the waves are not large, on account of the greater number of oscillations in the curve, due to the abnormal auricular activity.

We have pointed out that with five exceptions the modifications described were seen at a time when neither a change in rhythm or conduction time nor the onset of gastro-intestinal symptoms had taken place. An alteration in the curve may, therefore, be taken as a sign of the fact that an influence by digitalis is being exerted on the heart. The sign attains the greater importance on account of its appearance early after the beginning of the administration of the drug. We have detected changes in the T waves after an equivalent of 1.2 grams or even less of the dried leaves[3] of digitalis has been given, that is to say, on the third day of administration, although

[3] We have used digipuratum, a tablet of which represents 0.1 gm. of dried leaves. We usually give 0.4 gm. a day. The dose varies naturally with the patient's requirements. We have not estimated the size of the smallest dose capable of producing a change.

on several occasions we have seen the altered forms after thirty-six to forty-eight hours. On account of certain matters of technique, we have hesitated, in our earlier records, to recognize the onset of the alteration as early as it is quite apparent to us now that it occurs. The sign may, therefore, be looked on as one capable of detection earlier than the others, and as one indicating the fact that the drug is acting. Small changes measured in hundredths of seconds in the auriculoventricular conduction time occasionally occur as early as that noticed in the T wave, and when present may, of course, be employed as corroborating evidence of the fact that the drug is acting. The length of time required before the T wave is restored to its initial state varies. In the patient whose curves are reproduced in figure 6 twenty-two days elapsed before complete restoration occurred. In other cases it required only five days.

The influence of atropin on the T wave, after an alteration in it has become established, was tested more than forty times. Atropin sulphate was given either subcutaneously or intravenously in doses ranging from 0.9 to 1.8 milligrams, the size of the dose depending on the age of the patient and the method of administration. In every instance, the alteration which had taken place persisted after the drug was given. In several instances, when no change had been observed before atropin was injected, the characteristic modification appeared under the influence of the drug. Those alterations which atropin brought on or intensified almost always disappeared promptly; that is to say, before the next curve was taken, twenty-four hours later. Atropin alone, when no digitalis had been given, did not produce changes in the T wave. We have, however, an instance in which atropin lowered the isoelectric line between R and T slightly. Another modification in the outline of the digitalis curve occurs during the activity of atropin. The acceleration in rate which atropin causes is accompanied by a reduction in the time which elapses from the beginning of R (Q) to the end of T (figures 1 and 6 and text-figures 1 and 2). As a result of acceleration, the various elements which make up the ventricular electrocardiogram contract, but the essentials of the change which digitalis has brought about in the curve remain quite unaffected.

DISCUSSION.

In view of the fact that for human beings the form of the electrocardiogram is constant, and varies only because of distinct cause, the changes in it which have just been described must be looked upon as changes of significance. That they result from the action of digitalis must be concluded from the fact that they occur almost invariably when the drug is administered, and disappear more or less promptly when administration of the drug is discontinued. If the drug is given again to the same patient, after an interval during which the initial form of the electrocardiogram is restored, the same changes take place.

The interpretation of the changes in the T wave which have been described as occurring under the influence of digitalis presents difficulties, based on the interpretation of the formation of the electrocardiogram itself. If we adopt the current view, held by the earlier writers, Burdon-Sanderson and Page, Bayliss and Starling, and most of the later investigators, Einthoven, Samojloff, Garten, Boruttau, and Mines, that the cardiac action currents depend on electrical changes as an expression of muscular activity, then the changes in the T wave must be attributed to an alteration in muscular state under the influence of the drug. The fact that the T wave becomes negative indicates that the preponderance of electrical activity persists in the region near the apex of the heart longer than it does at the base; or, conversely, shorter at the base than at the apex. This is substantially the explanation that Samojloff and Boruttau adopt in explaining the action of vagus excitation and of muscarin. It opposes the view that persistence of electrical activity depends on a specific action directed toward a given part of the heart muscle, as Selenin supposed.[4] Greater electrical activity near the apex, therefore, accounts for apex negativity, and the consequent inversion of the T wave. The variations in the alteration of the terminal part of the wave already described depend on the exact site of longer electrical activity. If electrical activity at the base outlasts that at the apex, a diphasic wave results, the first portion of which is directed downward, corresponding to greater activity at the apex, and the second phase ends in a short upward deflection, corresponding to

[4] Selenin, *loc. cit.*, p. 154.

greater persistence of activity at the base (text-figure 1). Occasionally activity lasts longer, in the terminal phase of ventricular systole, at the apex, and this is shown by a diphasic wave, the direction of which in its first portion is upward, if the activity at the base is the greater, and in the latter portion downward (text-figure 2).

The observations of numerous pharmacologists on the action of digitalis bodies on the frog's and on mammalian hearts have demonstrated a diminished diastolic relaxation of the apical regions of the ventricles. In the later toxic stages of the action of the drug a systolic standstill of the apical regions is seen, while the base is still relaxing in diastole.[5] The changes we have observed in the T wave during the action of digitalis on the human heart may be evidence of a similar condition of the apical portion of the ventricles.

A fact to which we have called attention in these observations necessitates a possible modification in the view that the effect of digitalis is, so far as its influence on the T wave is concerned, wholly muscular. We refer to the fact that, on occasion, when a heart is subjected to the influence of digitalis, no change or only a slight one, takes place in the outline of the T wave. If atropin is injected under these circumstances, the alteration in the T wave, which was expected but failed to appear, becomes manifest immediately; the T wave either becomes inverted or becomes more negative than it was. When the influence of atropin terminates, the T wave returns to the state before injection. The view taken that atropin acts on the terminals of the inhibitory nerves implies, in this connection, that these exert an influence on the alteration in the wave, besides that attributable to the action of digitalis on heart muscle. Their influence, stimulated perhaps by the action of digitalis, is exerted against the full expression of increased electrical activity on the part of a given muscle area. When atropin is given, the inhibitor power is removed, and the full influence of digitalis on the muscle is manifest.

[5] This phenomenon is recorded by Cushny, A. R., A Textbook of Pharmacology and Therapeutics, or the Action of Drugs in Health and Disease, 4th edition, Philadelphia, 1906, 439. Asynchronous contractions of the frog's ventricle, due to strophanthus, are also described by Fraser, T. R., On the Kombé Arrow-Poison (Strophanthus cuspidus, D.C.) of Africa, *Jour. Anat. and Physiol.*, 1873, vii, 139.

These observations have yielded two-fold evidence bearing on the nature of the mechanism of digitalis action, considered from the point of view of whether this is muscular or nervous. We recall, in the first place, the difference between the effect of atropin on the T wave during digitalis, and during muscarin action. It has been shown that both drugs are capable of producing modifications in the T wave. The injection of atropin in the case of muscarin abolishes these, according to Samojloff and Boruttau, while in the case of digitalis the change persists or is intensified. The difference brought out between the two by the injection of atropin appears to depend on a difference in the site of their respective actions. The site of the action of muscarin on the cardiac inhibitors is well established;[6] its effect is consequently abolished when atropin, which acts on the nerve terminals, is given. It follows from this that if the inversion of the T wave under the influence of digitalis is not relieved by the injection of atropin, that influence is exerted at a site distal to the nerve endings; that is to say, on the heart muscle. We have shown that the inhibitors can prevent the formation or the full development of inversion in the T wave because it sometimes does not appear until after atropin is given. This curious phenomenon is exerted in opposition to the obvious muscular digitalis effect, and represents what appears to be a new phase of the function of the nerves. The second observation relating to the mechanism of the action of digitalis to which we have referred shows that it has two separate and distinct effects on the heart. The first consists in an alteration in the mechanism of the heart beat, a function which has been abundantly proved, and the second in the alteration of the T wave we are describing. The effect of the injection of atropin indicates that these two alterations depend on two activities of digitalis, quite distinct from each other, for the altered mechanism is abolished, while the altered T wave either persists or is intensified (figure 6). We attribute the altered heart rhythm which atropin is able to relieve to the action of digitalis on the inhibitory mechanism. The altered T wave which atropin fails to restore to its initial

[6] Recently the site of the action of muscarin has been reconsidered in papers by Straub, Samojloff, and Mines. Inasmuch as all agree that atropin has the effect mentioned, the argument advanced is considered valid.

state we attribute, as we have just shown, to the muscular action of digitalis. We assume for the purpose of this discussion that the effect of atropin is uniform; but we shall show in another place that this is true only within certain limits.

We wish finally to indicate the bearing that these observations have on the phenomenon of persistence of digitalis action. If alteration of the T wave depends on the action of digitalis, the time which elapses between the end of its administration and the return of the T wave to its initial state must be considered the measure of time during which digitalis is an active agent. The length of time required for restoration to the initial state has been seen to vary. In one observation it has been seen to take as little as five days; it has been observed to require as long as twenty-two days. The latter, if it is a common example of the duration of action, is a longer period for the drug to be active than is commonly supposed. Persistence of action may be compared with the duration of digitalis effect on rate in auricular fibrillation. In at least one such patient we have several times seen fourteen days elapse, after the administration of digitalis was stopped, before the ventricular rate began to rise. The statement (16) sometimes made that a second course of treatment with digitalis requires less of the drug to produce a given effect than the first is probably explained by our observation. For it is clear that if a second course is begun, before the first is completely terminated, a greater amount, equal to the residual quantity of the drug, must be present in the heart than had been expected.

SUMMARY.

It has been shown in this investigation that digitalis, administered orally to patients, can modify the T wave in the electrocardiogram. When the T wave in the initial curve is directed upward, the first change noticed is a lowering, and the final change is an inversion of the wave. It is not only the wave itself, but that portion of the curve between the end of R and the end of T which is involved. Instances in which the initial T waves have other than upright forms are described and their behavior under the influence of digitalis has been indicated. This influence of digitalis on the T wave may be

detected in thirty-six to forty-eight hours after the administration of digitalis has commenced; it may persist as long as twenty-two days after the administration has been stopped. Instances where it persisted only five days have been encountered. The unexpected length of duration of the sign probably explains why a second treatment with digitalis requires a smaller amount of the drug to produce the same effect, than the first.

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EXPLANATION OF PLATES.

In all figures divisions of the abscissæ equal 0.04 of a second; divisions of the ordinates equal 10^{-4} millivolts.

PLATE 59.

FIG. 1. A to H. The curves were taken from a patient who had a moderate grade of arteriosclerosis. A is the control. B, C, D, and E were made under the influence of digitalis; a few minutes before E atropin was injected. F and G show the gradual return to the form of the control. H substantially reproduces the control, A. The date to the left of each curve indicates when the curve was taken. The three usual leads (Einthoven) arranged in columns were obtained on each occasion. During the atropin test only lead 2 was made. The total amount of digitalis taken at the time each curve was made is given at the right.

PLATE 60.

FIG. 2. A to C. The curves were taken from a patient who had complete auriculoventricular dissociation. The three leads are arranged from above down. A is the control. B was taken after digitalis, 2.6 gm., was given. C substantially reproduces the control. The date on which each set of three curves was made is placed below, likewise the rate of the ventricles.

PLATE 61.

FIG. 3. A to D. These curves were taken from the same patient as those in figure 2. A is the control; B was taken under the influence of digitalis; C shows partial recovery; D substantially reproduces the form of the control curve.

PLATE 62.

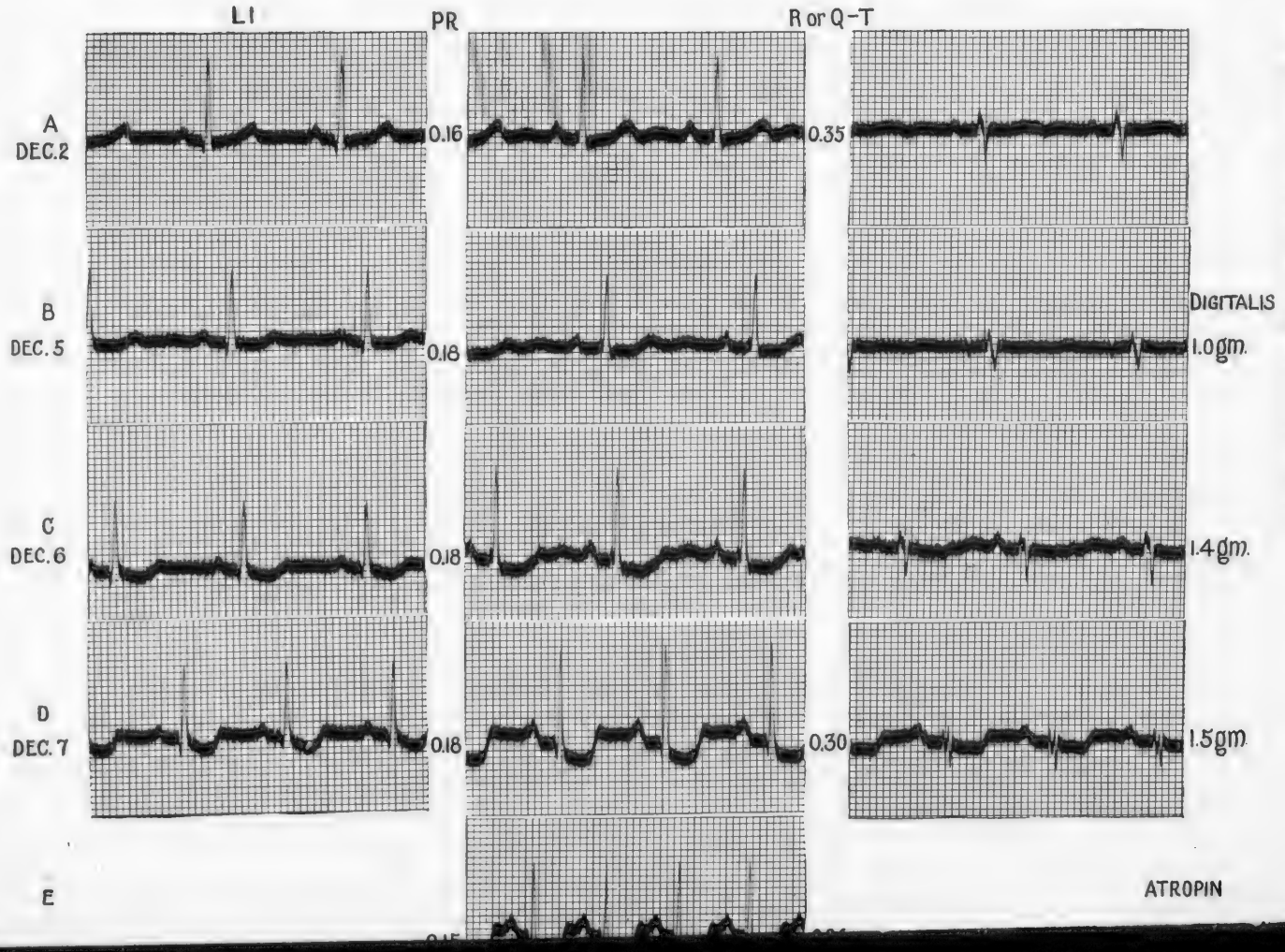
FIG. 4. A to L. These curves were taken from a patient who had mitral stenosis. The leads are arranged in columns. The date when each curve was taken is given at the left. A is the control. B, C, and D were taken under the influence of digitalis; the amount taken when the curve was made is given at the right. E to K shows the gradual return to the control. J, a single instance, breaks the gradation. L substantially reproduces the control curve.

PLATE 63.

FIG. 5. A to C. These curves were taken from a patient whose auricles were fibrillating. The leads are arranged as in figure 2. A was taken after digitalis, 3.2 gm., had been given. B is the control. C was taken after digitalis, 3.6 gm., had been given.

PLATE 64.

FIG. 6. A to C. These curves were taken from a patient who has mitral stenosis. The leads are arranged as in figure 2. A is the control. B was taken after digitalis, 2.5 gm., was given. The altered cardiac mechanism is described in the text. B, was made shortly after B, when atropin, 1.0 mg., had been injected. C substantially reproduces the control curve.


L I
PR
R or Q-T
A
DEC. 2
0.16
0.35
B
DEC. 5
0.18
DIGITALIS
1.0 gm.
C
DEC. 6
0.18
1.4 gm.
D
DEC. 7
0.18
0.30
1.5 gm
E
ATROPIN

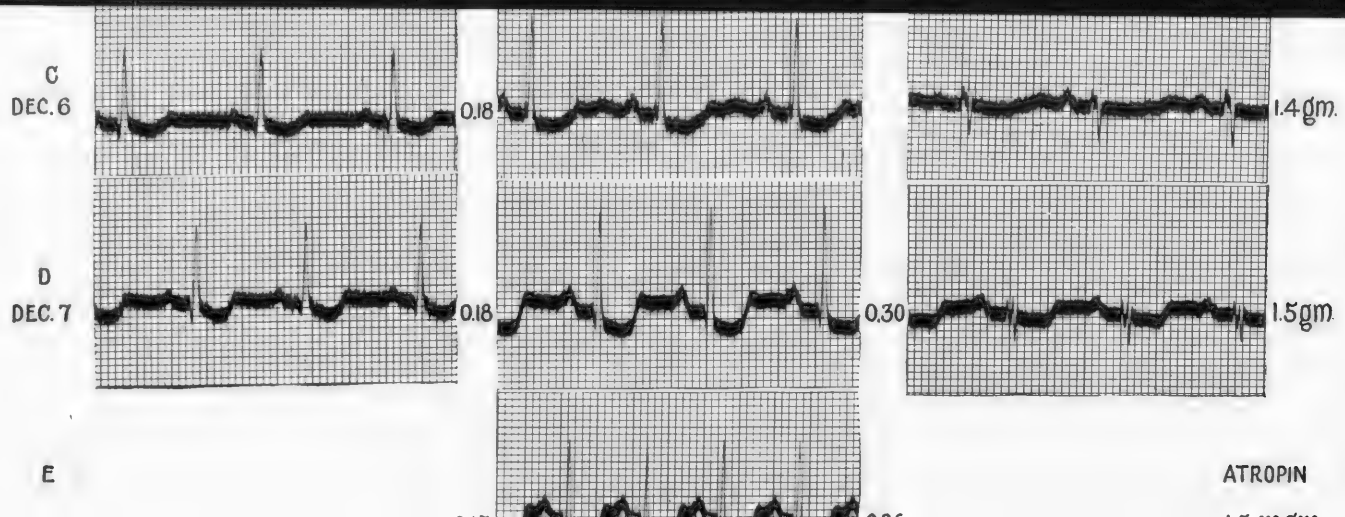

C
DEC. 6
0.18
1.4 gm.
D
DEC. 7
0.18
0.30
1.5 gm.
E
ATROPIN

FIG. 1.

(Cohn, Fraser, and Jamieson: Influence of Digitalis on Human Electrocardiogram.)

PLATE 59.

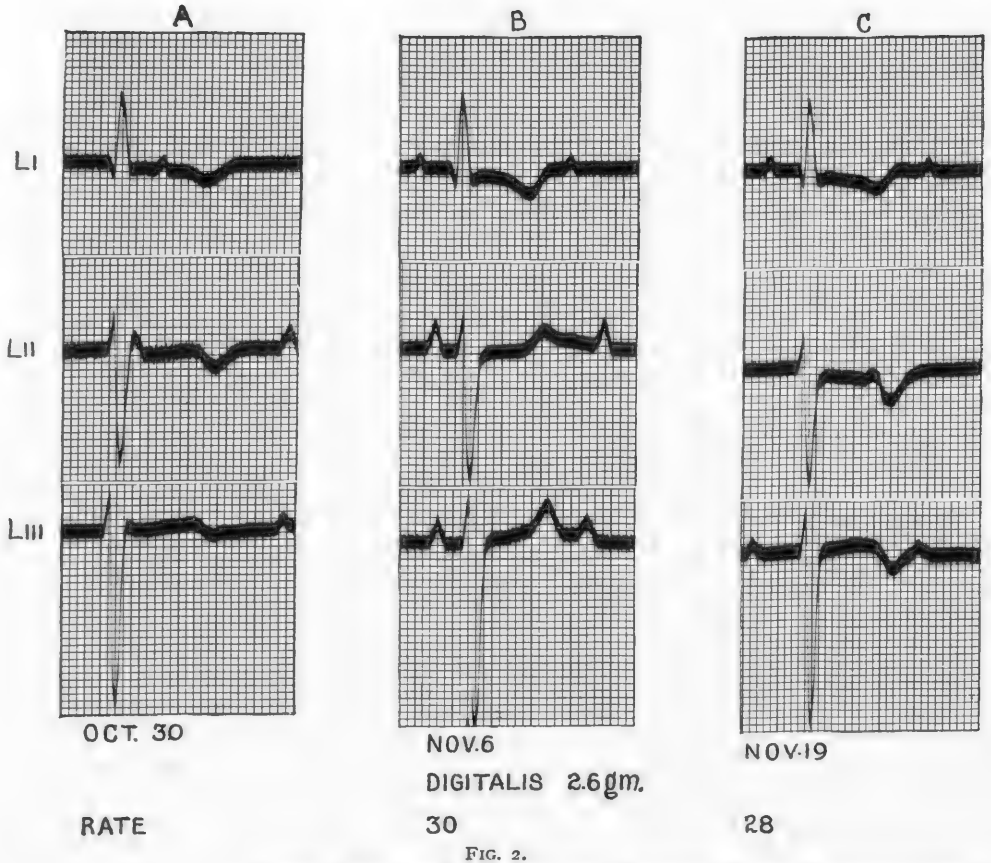


FIG. 2.

(Cohn, Fraser, and Jamieson: Influence of Digitalis on Human Electrocardiogram.)

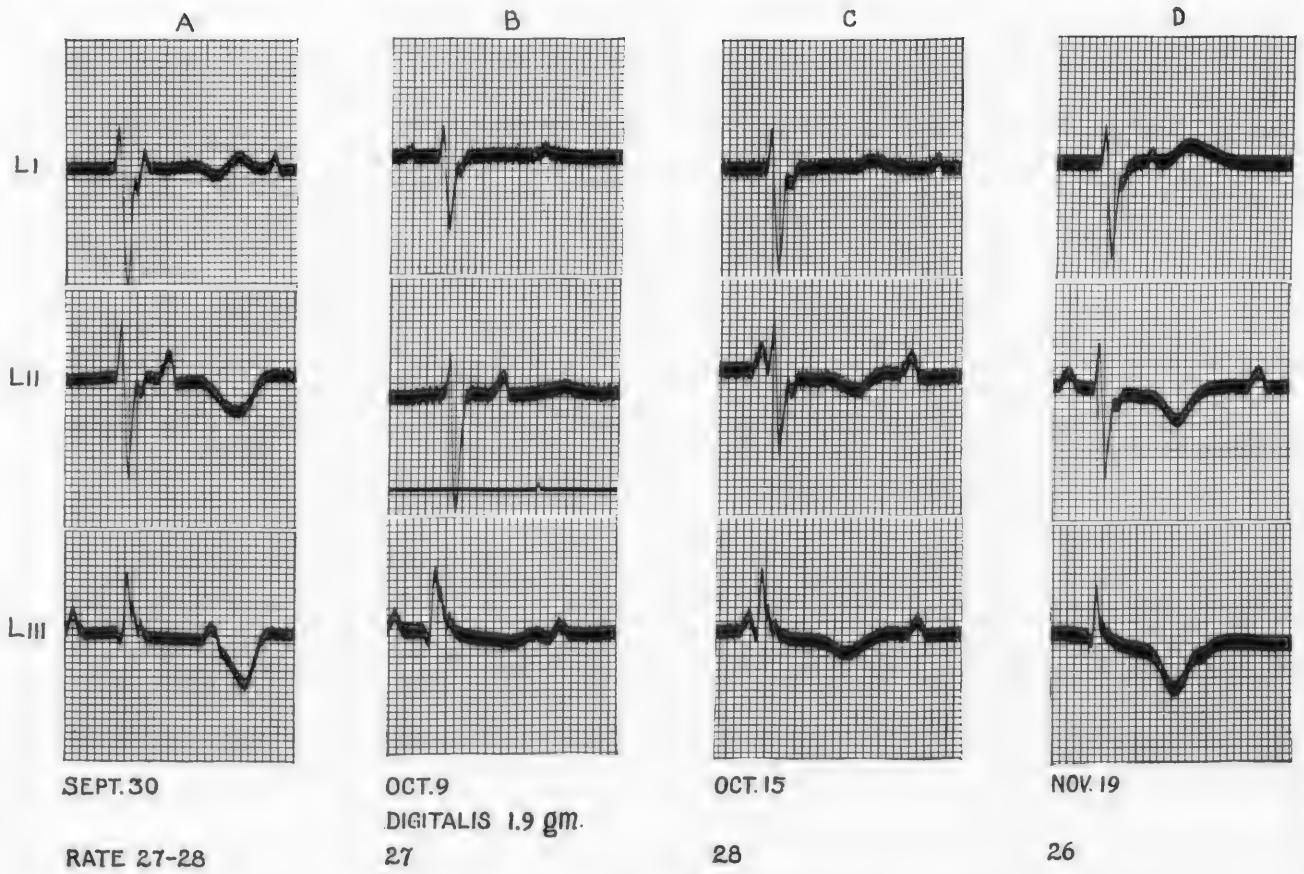
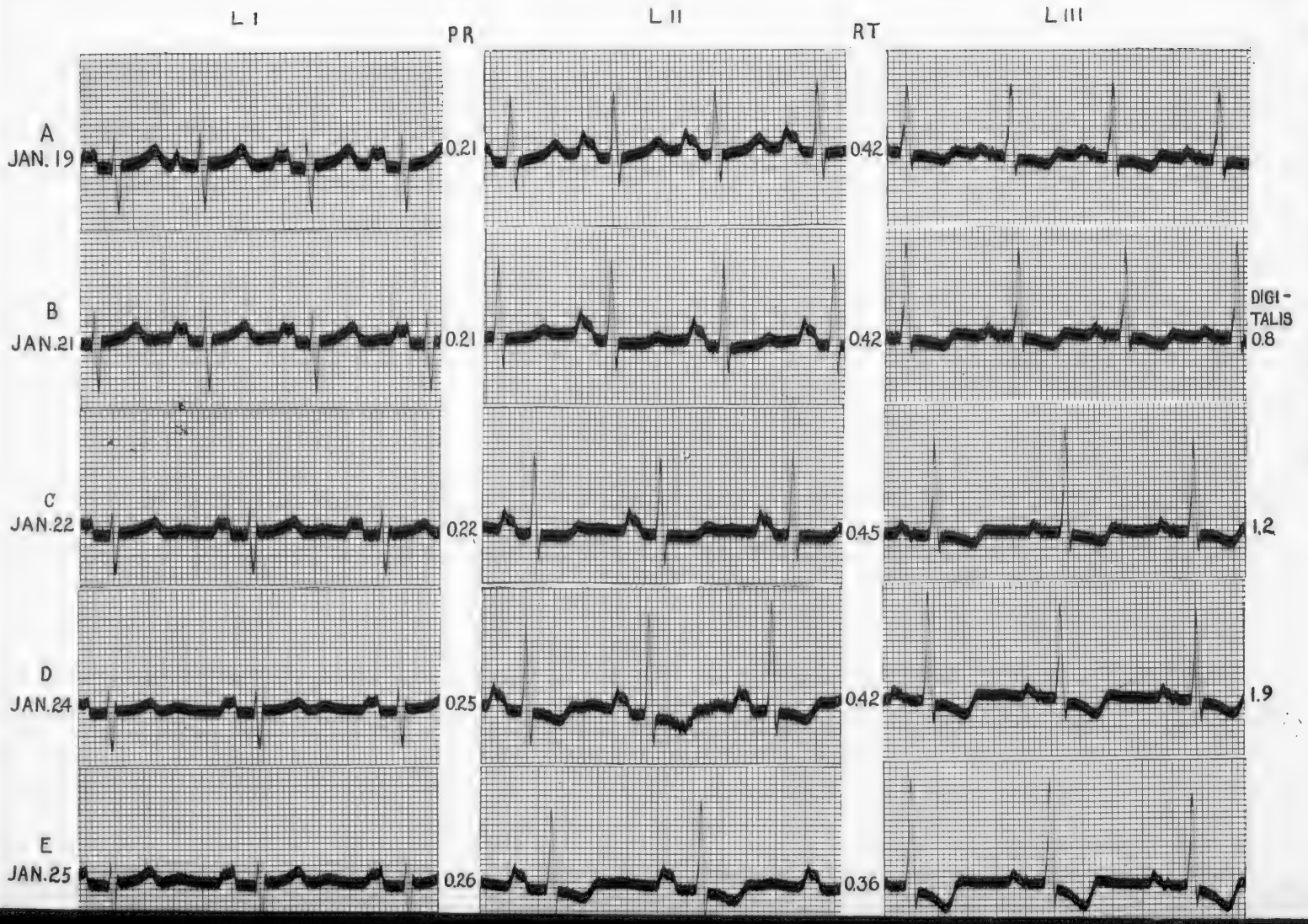


FIG. 3.

(Cohn, Fraser, and Jamieson: Influence of Digitalis on Human Electrocardiogram.)


L I
PR
L II
RT
L III
A
JAN. 19
0.21
0.42
B
JAN. 21
0.21
0.42
DIGI-
TALIS
0.8
C
JAN. 22
0.22
0.45
1.2
D
JAN. 24
0.25
0.42
1.9
E
JAN. 25
0.26
0.36

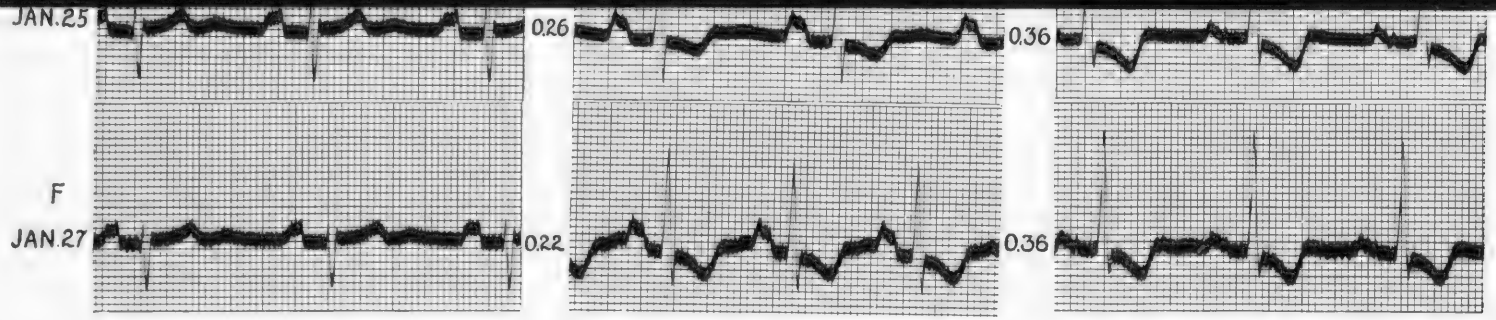

JAN.25
0.26
0.36
F
JAN.27
0.22
0.36

FIG. 4.

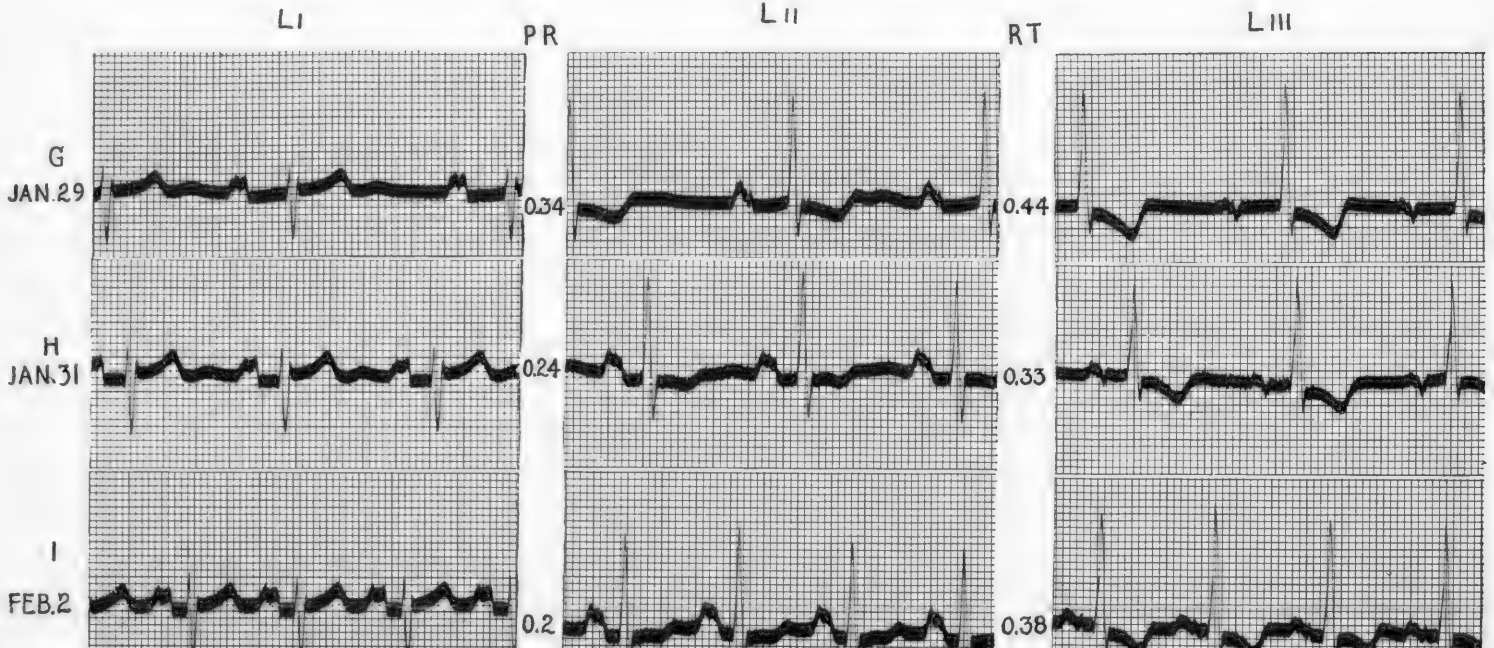

L I
PR
L II
RT
L III
G
JAN.29
0.34
0.44
H
JAN.31
0.24
0.33
I
FEB.2
0.2
0.38

FIG. 4 A.

(Cohn, Fraser, and Jamieson: Influence of Digitalis on Human Electrocardiogram.)

PLATE 62.

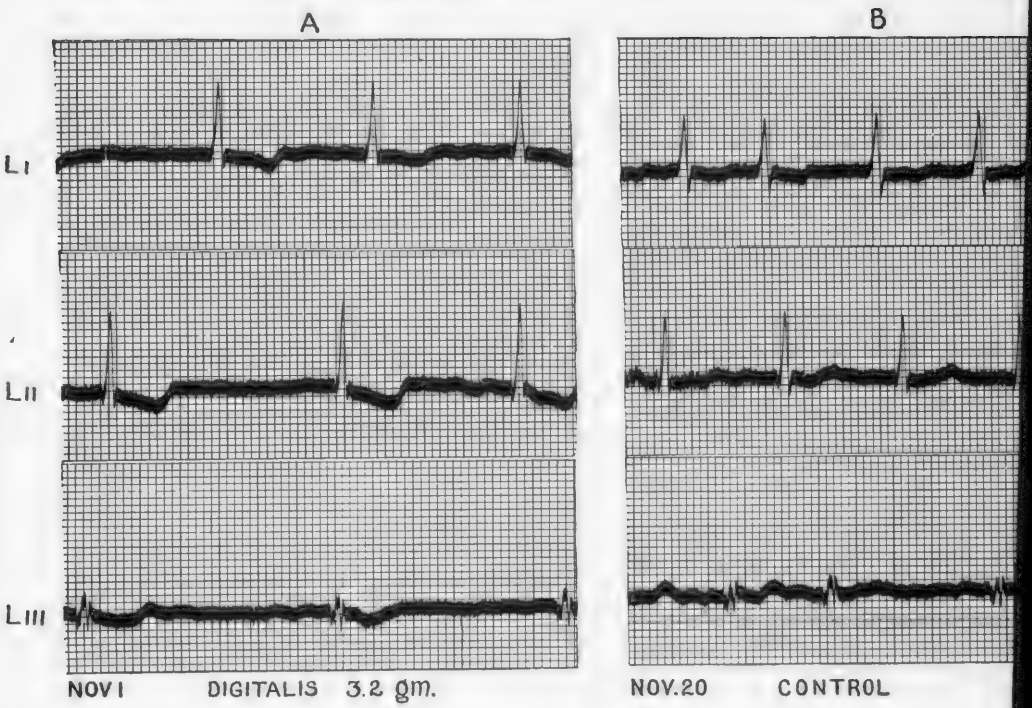


FIG. 5.

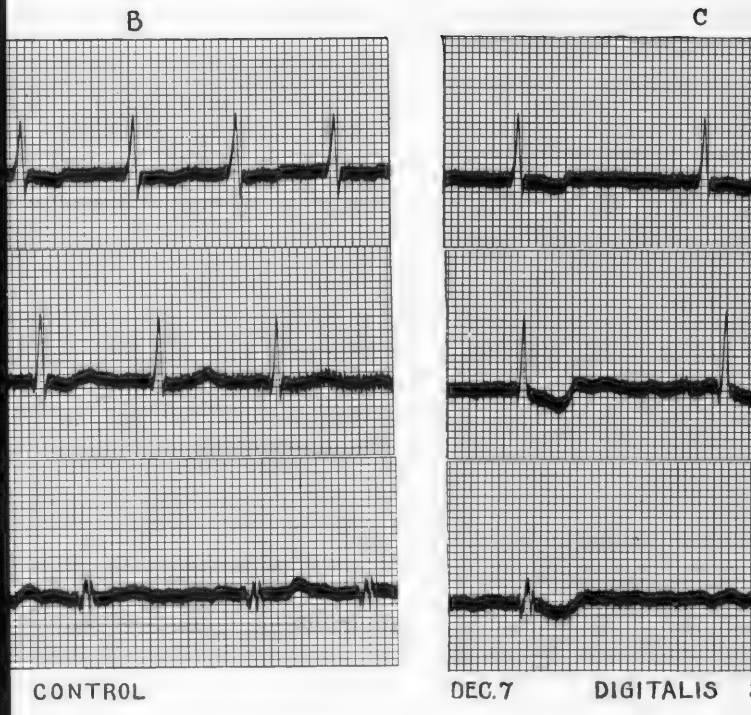
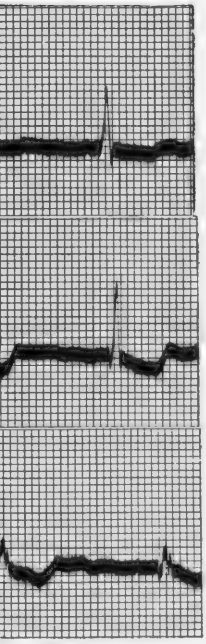


FIG. 5.

(Cohn, Fraser, and Jamieson: Influence of Digitalis on H

PLATE 63.



.6 gm.

man Electrocardiogram.)

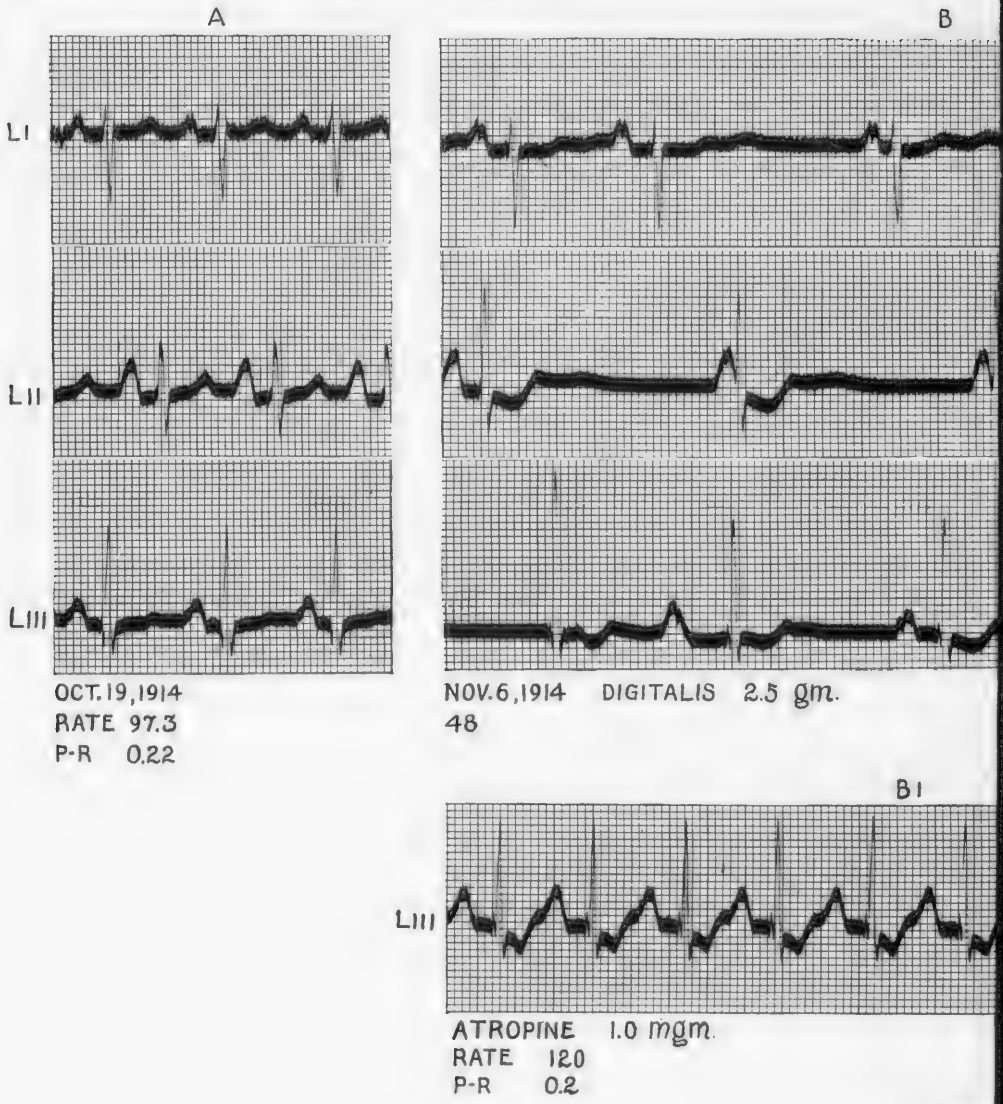
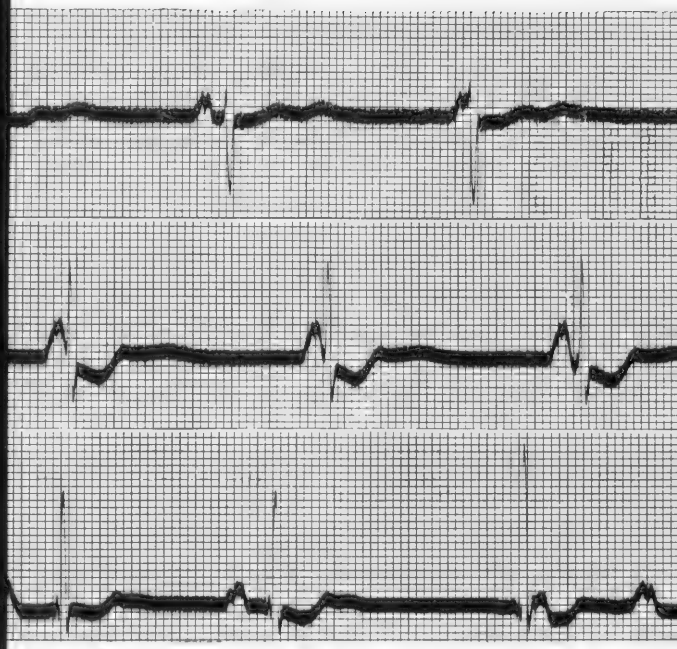

A
B
LI
LII
LIII
OCT. 19, 1914
RATE 97.3
P-R 0.22
NOV. 6, 1914 DIGITALIS 2.5 gm.
48
B1
LIII
ATROPINE 1.0 mgm.
RATE 120
P-R 0.2

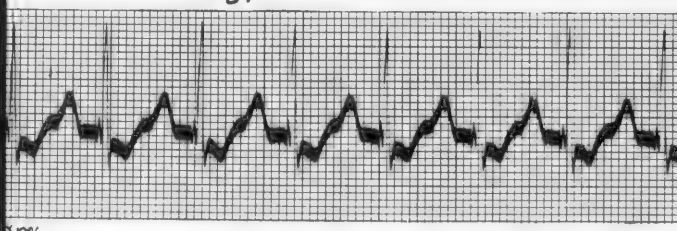
FIG. 6.

B



LIS 2.5 gm.

B1

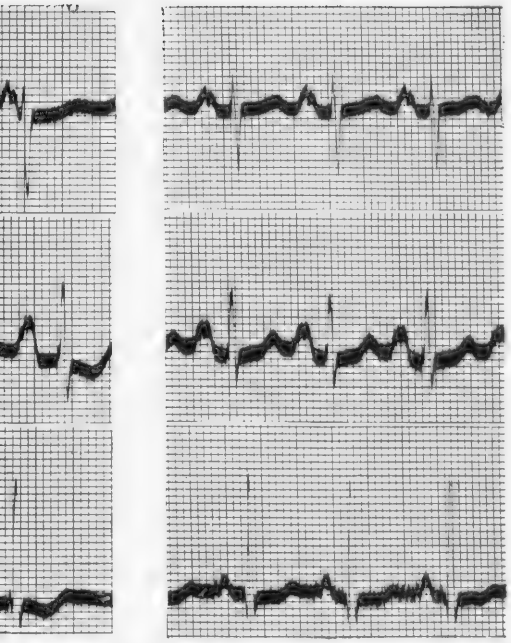


gm.

FIG. 6.

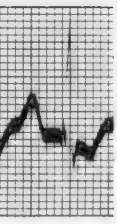
(Cohn, Fraser, and Ja

C



DEC. 29, 1914

0.2



(ieson: Influence of Digitalis on Human Electrocardiogram.)

A STUDY OF THE EFFECT OF SENSITIZATION ON THE DEVELOPMENT OF THE LESIONS OF EXPERIMENTAL PNEUMONIA IN THE RABBIT.*

By MARY BUTLER KIRKBRIDE.

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The analogies between the phenomenon of anaphylaxis in the laboratory experiment and the development and course of spontaneous pneumonia in man are, perhaps, more striking than those shown by any other infection. In pneumonia, moreover, conditions of hypersusceptibility might conceivably be one of the factors or the dominant factor, not only in the systemic febrile reaction but also in the localized and unique lung reaction such as is found only in man. The following study records a series of experiments in which an attempt was made to ascertain whether any definite and constant relationship between conditions of hypersusceptibility and the development of pneumococcus lesions of the lobar type in animals could be established.

Wadsworth,[1] in 1904,—before the phenomenon of anaphylaxis had been associated with infections,—stated that typical lobar lesions developed in a considerable number of partially immunized rabbits after tracheal injection of small quantities of pneumococcus culture. These lesions, absent when normal rabbits had been injected, were also much less extensive or lacking when highly immunized animals were used. Viewed from our present knowledge of anaphylaxis the acute lung reaction might possibly have been due to a state of hypersusceptibility unintentionally caused while attempting to induce a slight immunity as a predisposing condition to the development of the lobar type of lesion.

Typical lobar pneumonia has been successfully incited in the dog by Lamar and Meltzer[2] and others by intrabronchial insufflation of large quantities of pneumococcus culture. In rabbits the reaction has been induced experimentally with difficulty, as, owing to their extreme susceptibility to pneumococcus infection, a general bacteremia without characteristic lung involvement is usual.

* Aided by a grant from The Rockefeller Institute for Medical Research. Received for publication, November 5, 1914.

[1] Wadsworth, A., *Am. Jour. Med. Sc.*, 1904, cxxvii, 851.

[2] Lamar, R. V., and Meltzer, S. J., *Proc. Soc. Exper. Biol. and Med.*, 1909–10, vii, 102; *Jour. Exper. Med.*, 1912, xv, 133.

Rasquin[3] stated that tracheal injection of pneumococci gave typical pneumonias in only 7 to 8 per cent. of his rabbits, but when serum from a dog immunized to rabbit serum was added to the culture, 96 per cent. developed typical catarrhal lesions. Winternitz and Hirschfelder,[4] however, by tracheal insufflation of large amounts—4 to 5 c.c.—of concentrated culture were able to obtain extensive fibrinous involvement in a large proportion of fairly small rabbits.

Friedberger[5] and later Schlecht and Schwenker[6] reported a cellular bronchopneumonia produced by spraying horse serum into the tracheas of sensitized guinea pigs. Anaphylactic shock and pneumonic changes in the lungs of sensitized guinea pigs following intratracheal injection of small amounts of horse serum also have been reported by Ishioka.[7]

While the rabbit has proved more resistant to sensitization than the guinea pig, and the results generally less constant and less marked, definite anaphylactic reactions with bacterial proteins have been obtained. Neufeld and Dold[8] and Rosenow[9] incited acute toxic symptoms in the rabbit similar to those of anaphylaxis by using a pneumococcus culture and normal or immune serum-complement mixture, such as Friedberger had obtained with other bacteria. The toxic split products of pneumococci obtained by Vaughan, the autolysates of Rosenow, and the lytic bile extracts of Cole have all caused acute toxic reactions in the rabbit. Apparently in none of the experiments with bacteria has the toxic dose been given tracheally, nor has the local reaction called forth by it been studied.

In the present study two hypotheses have been kept in mind: one which would be strengthened by negative results, that in lobar pneumonia, notwithstanding its extraordinarily rapid and exceptional course, the progress is still a definitely progressive one and not dependent on conditions such as give rise to the sudden and eruptive phenomena of anaphylaxis; the second, suggested by the abrupt onset and sudden termination of the disease, by certain local reactions, and by the not infrequent clinical history of previous infection suggestive of sensitization, that its unique and striking features are of an anaphylactic nature. In the latter case pneumonia may be considered either as mainly a bacteremic or toxemic condition, the lung lesions being a local and comparatively unimportant feature of the disease as a whole, and the anaphylactic reaction a general and systemic phenomenon. Or, the lungs with their system of tubes

[3] Rasquin, E., *Arch. de méd. expér. et d'anat. path.*, 1910, xxii, 804.
[4] Winternitz, M. C., and Hirschfelder, A. D., *Jour. Exper. Med.*, 1913, xvii, 657.
[5] Friedberger, E., *Deutsch. med. Wchnschr.*, 1911, xxxvii, 481.
[6] Schlecht, H., and Schwenker, G., *Deutsch. Arch. f. klin. Med.*, 1912, cviii, 405.
[7] Ishioka, S., *Deutsch. Arch. f. klin. Med.*, 1912, cvii, 500.
[8] Neufeld, F., and Dold, H., *Berl. klin. Wchnschr.*, 1911, xlviii, 55.
[9] Rosenow, E. C., *Jour. Infect. Dis.*, 1911, ix, 190.

and air spaces may provide a particularly favorable environment and may act as a coöperating test-tube in which the cell complex furnishes the antibody, and the invading pneumococci the toxic agent. The resulting toxic substances, acting locally, might then incite exudative reactions in the lung, and on being absorbed give rise to the characteristic febrile reaction, the bacteremia being a secondary and transitory phenomenon.

Methods.—The pneumococcus organisms used were A, a virulent strain (the Neufeld culture), obtained through the kindness of Dr. Cole of the Hospital of The Rockefeller Institute; B, one of moderate virulence, but which had given evidence of unusual toxicity; and AA, an avirulent culture of A, attenuated by long continued growth on agar. Derivatives of eighteen to twenty-four hour meat infusion broth cultures were used for sensitization. The culture was centrifugalized and the supernatant fluid passed through a Berkefeld and then a Pasteur filter. The sediment was washed twice, suspended in salt solution equal to one-half or one-quarter of the original culture, and heated for thirty minutes at 52° to 55° C. Usually 0.1, 0.5, 7.5, and 15 cubic centimeters of filtrate or dead cell suspension were given intravenously to groups of rabbits. Two weeks later one cubic centimeter of eighteen to twenty-four hour live broth cultures was given tracheally to the rabbits. The same or a larger volume of concentrated suspension of living pneumococci cultivated aerobically in broth, or anaerobically under oil, or in large Petri dishes, was later used in a second series of rabbits. The effect of repeated sensitizing or toxic doses was also tested.

In studying the reactions incited by tracheal injection of serum and culture, mixtures were made of one cubic centimeter of live broth culture, and 0.1 or 0.5 of a cubic centimeter of sera from normal rabbits, or from animals immunized with pneumococcus filtrates, or with dead cells. These mixtures were usually injected immediately, and after incubation at 37° C. for periods of one to four hours.

The rabbits were etherized lightly and at the time of the tracheal injection tilted so that the natural course of the fluid would be into the left lung. Temperatures were taken before tracheal injection and once or twice a day afterward. If not already dead, the ani-

mals, with few exceptions, were killed at the end of forty-eight hours. At autopsy smears were made from the heart's blood and cultures from the pleural cavity, heart, liver, spleen, and trachea. Paraffin sections were made of all lungs, which had been previously distended and preserved in 95 per cent. alcohol or 10 per cent. formalin.

In the first series of experiments preliminary intravenous injection of culture filtrates or dead cells for purposes of sensitization was followed after an interval of two weeks by tracheal injection of living organisms.

SENSITIZATION WITH CULTURE FILTRATES.

Experiment 1.—A series of 20 rabbits received intravenously 0.1, 0.5, 7.5, or 15 c.c. of culture filtrate of the attenuated culture AA. Two weeks later 1 c.c. of a living broth culture of the same avirulent organisms was injected tracheally. None developed symptoms of anaphylaxis and all were killed after 48 hours.

While there was considerable variation in the lung reaction, in no instance was the lobar type of lesion approached, nor was there any apparent connection between the amount of the preliminary inoculation and the extent and nature of the reaction.

Experiment 2.—Similar preliminary treatment of 8 rabbits with filtrates of the same avirulent organisms, but with the virulent culture substituted in the tracheal injection, resulted in even less lung involvement.

Experiment 3.—In a third series 6 rabbits received both sensitizing and tracheal injections of the moderately virulent, though highly toxic strain B. The febrile reaction was acute. Only one rabbit died within 48 hours, although the lung involvement was much more diffuse and extensive than in the other series. The reaction, however, was apparently independent of the amount of filtrate previously received, and the unsensitized controls developed similar though, on the whole, less marked reactions.

Experiment 4.—In striking contrast was the almost complete absence of diffuse exudative lesions in the series of 16 rabbits receiving filtrates of the virulent strain A followed by tracheal injection of live organisms of the same virulent strain. The animals given the larger sensitizing doses had acquired considerable immunity, as they were still alive after 48 hours, whereas those sensitized with similar doses of the avirulent strain in experiment 2 died.

SENSITIZATION WITH DEAD PNEUMOCOCCUS CELLS.

Experiment 5.—19 rabbits which had received 0.1, 0.5, 7.5, and 15 c.c. of a killed suspension of the avirulent organisms AA were injected tracheally with 1 c.c. of a live broth culture of the same avirulent culture.

While there was more congestion and somewhat more lung involvement than in the corresponding filtrate series, the reactions did not differ materially in the different groups.

Experiment 6.—Eight additional rabbits which had been treated with the same dead cell suspension of the avirulent culture, but injected tracheally with the virulent organisms of the same strain, failed to develop marked exudative lesions. Treatment with large doses of dead cells had apparently a stronger protective action than similar treatment with filtrates of the avirulent culture, as two rabbits in this series were alive after forty-eight hours.

Experiment 7.—The reaction incited by the moderately virulent strain B, when given in both sensitizing and tracheal injections, was studied in 12 rabbits. The majority developed extensive exudative lesions in which areas of hemorrhage and necrosis were quite numerous. In four unsensitized controls, similar, but on the whole less extensive, areas of consolidation were found. The lesions, while more marked, closely resembled those in the corresponding filtrate series.

Experiment 8.—In this experiment in which sensitization with dead cells of the virulent culture was followed by tracheal injection of the same strain in the virulent state, 4 out of 14 rabbits developed extensive exudative lesions, in which fibrin and polymorphonuclear leucocytes were abundant. These lesions, however, were not confined to rabbits which had received the same sensitizing doses. While the two untreated controls died in 24 hours without marked lesions, the protective action of the sensitizing injections was shown by the survival for at least 48 hours of a number of the treated rabbits which had received the larger doses.[10]

In the following series concentrated cultures and culture material were used under similar conditions of experiment.

Experiment 9.—Of 16 rabbits treated with the usual concentration of dead cells, 4 received intravenous, and 12 tracheal injections of a concentrated suspension of living virulent organisms grown on agar. None showed anaphylactic symptoms. The lung reaction when present resembled that usually associated with foreign body pneumonias.

Experiment 10.—17 rabbits which had already received sensitizing and toxic injections were reinjected tracheally with a concentrated suspension of pneumococci grown in broth anaerobically under oil. No extensive lesions developed.

Experiment 11.—8 rabbits received single or repeated sensitizing injections of dead cells followed by intravenous injections of a concentrated suspension grown on agar. In one rabbit which had received a single sensitizing injection of 5 c.c., the toxic dose of 2 c.c. was immediately followed by symptoms resembling marked anaphylactic shock followed by complete recovery in about 30 minutes. The lungs at autopsy, 48 hours later, showed practically no involvement. In another series of 7 animals, one rabbit sensitized with 5 c.c. of concentrated dead cells and reinjected with 3 c.c. of a concentrated suspension of live organisms grown on agar died in less than 5 minutes, with symptoms resem-

[10] Additional sensitized rabbits in this or other series died in the latter part of the interval before tracheal injection; a number died of an infection present at the time, but less fatal to normal rabbits, suggesting that the animals, while they had acquired more tolerance to the pneumococcus, were so injured that they had become more susceptible to other infections.

bling anaphylaxis. No unusual reaction was found in the lungs on microscopical examination.

Experiment 12.—For the purpose of comparison 12 rabbits were sensitized with repeated injections of horse serum and reinjected at intervals intravenously or tracheally with the same serum. Anaphylactic shock developed in most of the animals receiving the toxic injection intravenously. Of those injected tracheally one was prostrated immediately after the injection, but recovered in half an hour. Killed 48 hours after the last tracheal injection. The sensitized rabbits' lungs showed possibly more peribronchial reaction than the unsensitized controls which had received tracheal injection, but the difference was not striking and no diffuse lesions of the lobar type were found.

In the preceding experiments active sensitization with filtrates or dead cells of strain A in its highly virulent or avirulent state followed by tracheal injection of live virulent or attenuated cultures of the same race did not result in a definitely increased lung involvement. When a second strain B, of much less virulence, was used, marked exudative lesions, varying to some extent, developed in the rabbits previously sensitized with filtrates or dead cells of the same strain, but the untreated controls also developed similar though possibly less extensive reactions. Since these experiments with active sensitization failed to bring out any very definite relationship between sensitization and the character of the lung lesions, the effect of passive sensitization by means of tracheal injection of different sera and culture mixtures was next studied.

TRACHEAL INJECTION OF MIXTURES OF SERUM AND CULTURE.

Experiment 13.—Mixtures of 0.5 c.c. of fresh pooled normal rabbit sera and 1 c.c. of living attenuated culture, freshly mixed or incubated, were given tracheally to 8 rabbits. The animals almost at once developed marked signs of discomfort, such as restlessness, dyspnea, and prostration, their temperatures falling at least 3.2° F. The control rabbit, however, receiving 1 c.c. of culture diluted with 0.5 c.c. of sterile broth developed similar, though less marked, symptoms. All recovered in half an hour. The control and the rabbit receiving the mixture incubated for over 3 hours died in about 48 hours. The seven others died within 4 hours of each other in sudden and violent paroxysms, about 24 hours after the tracheal injection. Pneumococci were found in all cultures. A small rabbit injected intravenously with 1 c.c. of a transfer from the culture used in this experiment was unaffected.

The control's lungs showed some peribronchial infiltration. Two rabbits which had received serum mixtures developed extensive, two less marked diffuse fibrinous lesions. Repetition of the experiment failed to incite unusual symptoms, though in the lung of several rabbits small lesions of a fibrinous character were found.

Experiment 14.—Similarly mixtures of normal rabbit sera and virulent living culture were given tracheally to 21 rabbits. No immediate symptoms developed, but a number died in acute paroxysms 24 hours after tracheal injection. In the first series of these rabbits receiving a mixture containing 0.5 serum, which had stood at room temperature for about 2½ hours, one rabbit developed a typical fibrinous lobar pneumonia, the other smaller diffuse lesions. In later series the proportion of rabbits developing diffuse and fairly extensive lung involvement was, however, much smaller. Incubation of the mixtures for 2 to 3 hours appeared to favor a slightly increased lung reaction.

Experiment 15.—Of 12 rabbits receiving mixtures of avirulent living culture and sera from rabbits immunized with culture filtrates of the virulent strain, none showed immediate symptoms, but 7 died within 24 hours of injection—the majority in violent convulsions. The control which had received culture without serum died while not under observation in less than 48 hours. While four serum mixture rabbits developed considerable diffuse fibrinous involvement, the remainder, including the control, showed little or no exudative reaction.

Experiment 16.—The effect of virulent cultures and sera from rabbits immunized with filtrates of the same virulent strain A was tested on 26 rabbits. Of four receiving mixtures which had stood at room temperature 2 to 3 hours and in which the sera was three weeks old, two developed typical fibrinous lobar involvement. In the other series one rabbit showed extensive, and quite a number smaller diffuse fibrinous lesions. While the animals receiving the larger amount of sera possibly showed more lung reaction, the period of incubation apparently exerted little or no effect in this experiment.

Experiment 17.—The experiment was repeated, 11 rabbits receiving attenuated cultures and sera from animals immunized to dead pneumococcus cells. As before, the mixtures were incubated for varying periods. All the rabbits were still alive at the end of 48 hours. The lung reaction was distinctly less than in the corresponding normal or filtrate sera and avirulent culture experiments.

Experiment 18.—A series of 22 rabbits was given similar mixtures of virulent culture and sera from rabbits immunized to dead cells. The sera would seem to have exerted a certain amount of protective action, as over half of the rabbits were alive at the end of 48 hours. None developed extensive lesions.

The effect of intravenous inoculation of normal and immune sera immediately before tracheal injection of live culture was tested in the following experiment.

Experiment 19.—11 rabbits were inoculated intravenously with 0.1 or 0.5 c.c. of normal rabbit or horse sera, or with sera from rabbits immunized with filtrates or with dead cells. This was immediately followed by tracheal injection of living virulent organisms. Of the three rabbits, which had received injections of sera from rabbits immunized with filtrates, all died practically 20 hours after injection in acute paroxysms. One of three rabbits, receiving sera from rabbits immunized with dead cells, was alive after 48 hours; the others died while not under observation. None developed diffuse lesions.

The distribution in the lungs of material injected through the trachea, and the resulting injury, must necessarily vary considerably. While these mechanical factors were undoubtedly responsible for certain differences in the bronchopneumonic reaction, they did not obscure the results. Tracheal injections of only 1 or 1.5 cubic centimeters had been given to avoid reactions caused by excessive quantities of concentrated culture such as Winternitz and Hirschfelder had obtained in normal rabbits, and also because it seemed desirable to approach more closely conditions of spontaneous pneumonia in man. Similarly, small sensitizing and non-concentrated toxic doses had been carefully tested, as it was thought a hypersensitive condition favorable to the development of a definite cellular reaction in the lung might be present, although acute or even mild anaphylactic symptoms were entirely lacking.

Active sensitization by previous intravenous injection of pneumococcus filtrates or dead cells failed either to hasten death or to increase markedly the lung reaction, although beginning with as small sensitizing doses as 0.1 of a cubic centimeter the amount injected had been increased until there had been produced an immunity sufficient to protect. In fact, the controls which had not received preliminary treatment were usually the first or among the first to die, indicating that the chief effect of the preliminary treatment was to increase materially the resistance of the animals, especially when large doses were used. While filtrates of the non-virulent culture AA, although originally derived from the extremely virulent strain A, failed, even in large doses, to protect from tracheal injection of virulent organisms of the same strain, and while the protective action of the moderately virulent strain B could not be gauged, as both controls and treated rabbits, with one or two exceptions, live for forty-eight hours, in no instance was death hastened as a result of sensitization.

Comparison of the histological changes in the lungs of treated rabbits and untreated controls also failed to show striking or constant differences, though it appeared that preliminary treatment, especially with dead cells, possibly favored a somewhat more diffuse reaction. In the two experiments in which extensive lesions were found, not only in the treated animals but also to a lesser extent

in the untreated controls, the strain used, though of moderate virulence, was considered especially toxic as compared with the more virulent strains studied. Extremely virulent organisms, on the other hand, apparently encountered little resistance from the lung tissues, but passed immediately into the circulation without inciting exudative reactions. In certain instances where large preliminary doses had been given, the lung involvement was also proportionately greater.

These results would seem to bear out in some measure the view that in pneumonia the lung reaction is partly dependent on a favorable equilibrium obtaining between the natural or acquired resistance of the host and the degree of virulence and toxicity of the organisms rather than on an anaphylactic state.

The reactions, both systemic and local, incited by tracheal injection of serum culture mixtures were somewhat more marked. A supposedly attenuated culture to which normal sera had been added caused immediate but transient symptoms suggesting mild anaphylactic shock. Seven of the eight rabbits, however, died in violent convulsions twenty-four hours later. These acute paroxysms, which closely resembled those of fatal anaphylaxis, also occurred in other series about twenty to twenty-four hours after serum mixtures had been injected.[11] The animals suddenly showed extreme restlessness, threw themselves about their cages, making violent running or jumping motions and often crying out. Death occurred within a few minutes of the onset of the symptoms, the heart continuing to beat for some time after respiration had ceased. Examination of the lungs failed, however, to show any definite connection between these paroxysms and the development of exudative reactions.

It is difficult to account for the sudden death of such a large number of the animals at practically the same time, although the interval after inoculation varied in the different experiments, except as the result of some phase of delayed anaphylactic shock. This delayed reaction might be due to several factors, but not to the tracheal method of injection alone, because similar reactions also

[11] In other experiments similar results were noted as early as 10 to 12 and as late as 32 to 34 hours after inoculation.

occurred after intravenous inoculation. The capsule of the pneumococcus cell which is protective according to Welch, and the interval required for the production of sufficient toxin, whether from the body fluids or from the pneumococcus cells through growth, might be important factors.

The local tissue reactions incited by the serum culture mixtures, particularly those containing normal serum or serum from rabbits immunized to filtrates, were also somewhat more marked and developed in a larger proportion of animals than in the experiments with active sensitization. Several rabbits developed typical fibrinous lobar pneumonia, and a number of others less extensive but diffuse exudative lesions. These were partly offset, however, by the partial or almost complete absence of exudative reactions in the other rabbits of the experiments, which had received similar treatment.[12]

Throughout the experiments the absence of uniformity in the lung reaction was especially striking. Controls in a few instances developed more involvement than certain of the treated rabbits of the same experiment, while in several series the test animals ran the whole gamut of reaction without apparent relation to the special character of preliminary treatment or tracheal injection.

The extreme sensitiveness of the pneumococcus to any alteration in its environment was repeatedly noticed. While the causes of fluctuation in virulence and toxicity are obscure, they not infrequently seemed closely associated with growth activity. That extremely subtle reactions occur within the body seems probable, and until the laboratory methods more nearly duplicate conditions present in the body, where the cellular elements and body fluids undoubtedly play an important part, similar and constant results can hardly be expected. In the present experiments when body conditions were more nearly approached the lung reaction was generally increased. It is of interest that in Wadsworth's experiment with partial immunization, the pneumococci used in the tracheal injection were grown in equal parts of normal rabbit serum and broth, while Winternitz and Hirschfelder used a 5 per cent. serum medium.

[12] For purposes of comparison tests with heterogeneous sera were made. Three rabbits received tracheally mixtures of culture and sera from a horse immunized to live cultures. Two developed diffuse and fairly extensive exudative lesions, one a very slight reaction.

Although an anaphylactic or hypersensitive condition in the host may possibly enable the invading pneumococcus to gain its first foothold, similar conditions might equally well underlie the development of a streptococcus infection, but would not necessarily determine in either case the type of lesion that later develops. In this study the essentially progressive character of the lung lesion was frequently brought out, all degrees of exudative reaction being found from small peribronchial foci merging into patchy or confluent lesions to the typically lobar involvement.

SUMMARY.

While preliminary treatment with culture filtrates or dead cells of the extremely virulent strain A gave rise to varying degrees of immunity, the exudative lung lesions developing after tracheal injection with live organisms of the same virulent strain were not strikingly increased in any group or series of these rabbits. Despite carefully graduated dosage in the preliminary treatment, none of the animals developed symptoms of a definitely anaphylactic nature.

In similar experiments following sensitization with the attenuated avirulent culture AA, tracheal injection of virulent or avirulent organisms of the same strain failed to incite any definite increase in the exudative lung reaction. In none of the rabbits were symptoms resembling anaphylaxis noted. The immunity which was induced by the larger sensitizing doses of culture filtrates of the strain in the virulent state was lacking when similar doses of the culture filtrates of organisms in the non-virulent state were used.

Extensive lesions developed in both sensitized and unsensitized rabbits when the strain used in the tracheal injection was one apparently combining moderate virulence with exceptional toxicity, indicating that the exudative lung reaction was one of adjustment rather than of acquired hypersusceptibility.

When, in the experiments, small amounts of sera from normal rabbits, or from animals immunized to culture filtrates, were added to the culture before tracheal injection, an increased fibrinous lung reaction was frequently found.

The present study would seem to give some ground for the view that while in pneumonia a hypersensitive condition probably takes

some part in the inception of the infection, the subsequent development of the diffuse exudative reaction in the lung is not directly due to an acquired hypersusceptibility, but to intrinsic qualities possessed by the pneumococcus itself.

This study was carried on in the Department of Bacteriology of the College of Physicians and Surgeons of Columbia University, New York, and later, through the courtesy of Dr. Charles Norris, in the Bellevue Hospital Laboratories. It was completed in the Laboratories of the New York State Department of Health at Albany. I am greatly indebted to Dr. Augustus Wadsworth for his advice and criticism.

THE VISUAL CORTEX, ITS LOCALIZATION, HISTOLOGICAL STRUCTURE, AND PHYSIOLOGICAL FUNCTION.*

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The cytoarchitectonic study of the cortex has reached a high development during the last twenty to thirty years. In earlier times it was believed that the whole cortex represented more or less a homogeneous organ, but it has since been concluded that this is not the case. Though the same architectonic ground type may be found in the entire cortex, yet in different regions certain histologically well characterized modifications of the cortex exist, with small variations, not only in man but in all higher mammals. In the most characteristic and well marked type of cortex belongs that portion called by Elliot Smith the area striata. On account of its special morphological character and its physiological importance, that is, its relation to the act of seeing, the area striata has been investigated with special attention. In this paper I shall discuss its localization, anatomical structure, and physiological function.

It was known to the earlier investigators, Gennari, Vicq-d' Azyr, and Baillarger, that the cortex of the fissure calcarina had a different structure from the rest of the cortex. One can readily see with the naked eye in the gray substance a stripe of white fibrous matter lying tangentially. This is called the stripe of Gennari or of Vicq-d' Azyr. The characteristic marks of the morphology and order in this part of the cortex were recognized much later by Meynert. A number of authors (Betz, Leonowa, Hammarberg (1), and Schlapp) have partly abandoned the division of layers after Meynert, but they have given no new point of view. They have also distinguished eight layers in the cortex. Cajal, who has studied the cortex with the help of the chrome-silver method of staining, has distinguished nine layers in the division of the visual cortex, based principally on the fine morphology of the cells.

Brodmann (2) has given the most detailed study of the cortex in general and especially of the calcarine type. He has not only studied each type histologically, but has defined its extension topographically on the surface of the

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cortex and has produced what may be called brain maps. The two most characteristic types of the cortex are the giant pyramid types, called the area gigantopyramidalis, and the calcarine type, named area striata. He has studied them in their variations and extensions in man and primates, and also in most classes of mammals. I shall keep chiefly to his descriptions in what follows, so that the connection of each layer in the area striata, to the sixth layer ground type, appears distinctly as six fundamental primary types of the cortex. It seems to me that Brodmann is clearer on this point when he divides the area striata into six layers, of which the fourth is subdivided into three secondary layers.

I. Lamina Zonalis, or Moleculosis.—This is well marked; it contains single small nerve cells of different shape, partly round, partly star-shaped, with short points. It does not differ essentially in its construction from the first layer of the rest of the cortex.

II. Lamina Granularis.—The external granular layer, or layer of small pyramids. This is sharply outlined in the cortex of adults, especially at its junction with the lamina zonalis. It consists of a narrow layer of granular or small pyramidal cells lying closely together. The transition from the small to the medium sized pyramids of the third layer goes on gradually, so that often it is difficult to distinguish here two different layers as in the rest of the cortex; that is why it is often included with the third layer.

III. Lamina Pyramidalis.—The layer of the medium sized pyramids is also small and poorly developed. The pyramids do not reach, anywhere, the size of the neighboring area occipitalis. Already in the inner part of this layer appear small round nerve cells called granules, whose number increases continually; at the inside edge of the pyramid layer they undergo a condensation which Brodmann considers as a special layer.

IV. The Lamina Granularis Interna, Superficialis IVa.—According to Brodmann, the undivided inner granular layer of the neighboring type, is divided in the visual cortex into a superficial and a deep sublayer; between the two a layer, poor in cells, is enclosed, the lamina granularis intermedia IVb. This latter is especially characteristic of the visual cortex.

In the Weigert preparation is here seen the stria of Vicq-d' Azyr or of Gennari, a tangential white stripe visible with the naked eye.

After being stained by Nissl's method this layer is seen as a light stripe, poor in cells. Here are the characteristic, small, medium sized, and star-shaped single cells.

Next comes the principal granular layer IVc, the lamina granularis interna of Brodmann.

In comparison with the preceding layer this one appears well marked, and about as broad as IVa and IVb together. It consists of numerous granules lying close to each other; there are also single elements of the neighboring layer called "guests"; star-shaped, spindle-shaped, and pyramid-shaped cells.

V. The Ganglion Layer (Brodmann), or the Intermediary Layer (von Monakow).—This forms again a clear layer of single, scattered cells, which contain separate large pyramidal cells, named Meynert's solitary cells. In fiber preparations there is seen also a small stripe of transverse fibers, the under stria of Vicq-d' Azyr or Gennari.

VI. The Multiform Layer.—This layer, the layer of polymorphic elements of von Monakow, represents a clear and characteristic part, which is marked towards the outside by the lamina ganglionaris and towards the inside by the stripe of white matter or fibers. In spite of its narrowness it consists of two layers; an outside layer of compact, mostly three cornered cells, the lamina triangularis, and an inner layer of loose spindle-shaped cells, the lamina fusiformis.

It is therefore characteristic of the area striata of man that the large pyramidal cells in the pyramidal layer are missing. In the layer of star-shaped cells, the Gennari stripe and the large inner granular layer, also a well marked multiform layer rich in cells, and the closeness and smallness of the cells are characteristic of this type (text-figure 1). Brodmann studied the tectonic variations and the extension of the calcarine type of the cortex, not only in man but in all mammals. He worked out the homology of the area striata in all classes of mammals as far as they were examined. The area striata of the monkey is similar in structure to that of man. It is here also marked by a light layer of numerous star-shaped cells, and a broad inner granular layer in which star-shaped cells are scattered about.

In some monkeys, like *Cebus capucinus,* there is a different cyto-architectonic structure which surpasses that in man. Here, inside of the star-shaped layer, is another layer of larger cells running into each other, so that this layer can again be divided into three secondary layers: a superficial layer, a dark middle layer, and a deeper light one.

I. Lamina zonalis.

II. Lamina granularis externa.

III. Lamina pyramidalis.

IVa. Lamina granularis interna superficialis.

IVb. Lamina granularis intermedia; here lies the stria of Gennari.

IVc. Lamina granularis interna profunda, or principal granular layer.

V. Lamina ganglionaris.

VIa. Lamina triangularis.

VI. Lamina multiformis.

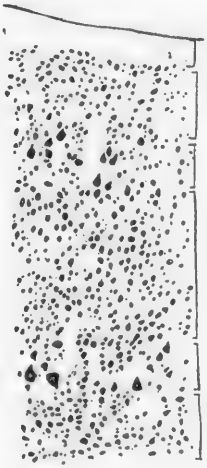
VIb. Lamina fusiformis.

TEXT-FIG. 1.[1] Calcarine cortex. Man. × 44.

[1] A list of the meanings of the abbreviations used in the text-figures is given at the end of the paper.

Besides the more advanced tectonic development of the calcarine cortex of some primates is the large number of mammals with a more or less undeveloped or retrogressed type (Brodmann). According to Brodmann, the insectivora are a highly undeveloped type.

In carnivorous animals (cat and dog) a special layer of star-shaped cells can not be distinguished (text-figure 2). The area stri-



I. Lamina zonalis.

II. Lamina granularis externa.

III. Lamina pyramidalis (very narrow).

IV. Lamina granularis interna. Broad and light layer. In Weigert preparation the striæ of Gennari are seen.

V. Lamina ganglionaris, with Meynert's large solitary cells.

VI. Lamina multiformis. Many cells with sharp outlines.

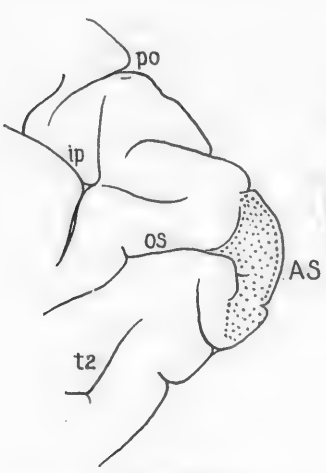
TEXT-FIG. 2. Calcarine cortex. Cat. × 40.

ata is here especially characterized by a broad and clear inner granular layer in which star-shaped cells are scattered about. We find, on the whole, that the other characteristics are the same as those of primates; that is, a narrow, light sixth layer (lamina ganglionaris) with some large pyramidal cells. In general the cortex is very narrow; it is more compact, and the size of the cells is smaller. The transition of the area striata to the surroundings is not so well marked as in man and primates, but a gradual transition takes place. The pyramidal cells of the third layer become larger and go more deeply into the cortex; the inner layer becomes more compact and smaller. The lamina ganglionaris and multiformis are less frequently seen, or, briefly speaking, the ordinary six layer type appears again.

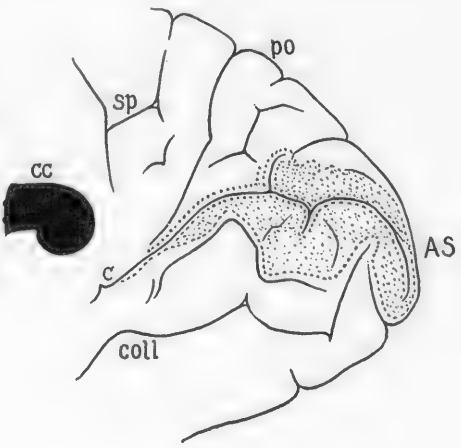
Until recently little was known about the exact localization of this type of cortex in man, and still less in animals. Bolton has de-

scribed (1900) the extension of the visuosensory area in the human cortex. His views were, in general, confirmed by Brodmann, Campbell, and others. It is now possible to give an exact division of this human cytoarchitectonic cortex field, as its boundaries are well marked in all directions.

Text-figures 3 and 4 show the extension of the area striata in the human occipital lobe (after Brodmann). Near the convexity it



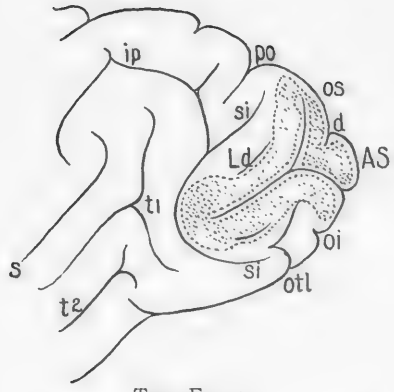
TEXT-FIG. 3.



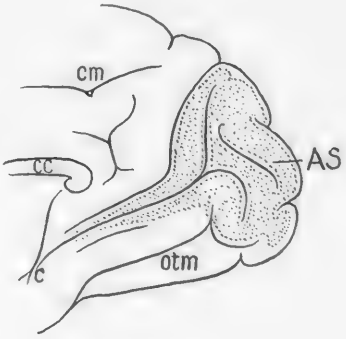
TEXT-FIG. 4.

TEXT-FIGS. 3 and 4. Extension of the area striata in the human occipital lobe, convex and mesial surfaces; after Brodmann.

occupies only the extreme caudal end. On the mesial surface of the hemisphere it occupies the two lips of the calcarina, also the fissure retrocalcarina, which is hidden inside of the occipital lobe. It reaches from here to the neighboring regions; dorsally to the cuneus, and ventrally to the lobus lingualis. Anteriorly it diminishes quickly and ends after joining the fissure calcarina and the fissure paroccipitalis in the posterior part of the trunks of the fissure calcarina. The transition of the area striata into the structural type of the surroundings takes place suddenly; dorsally into the cortex of the cuneus, ventrally into that of the lobus lingualis, and on the convexity into the area occipitalis. The layer of star-shaped cells disappears, the

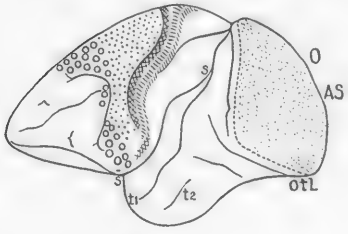


TEXT-FIG. 5.

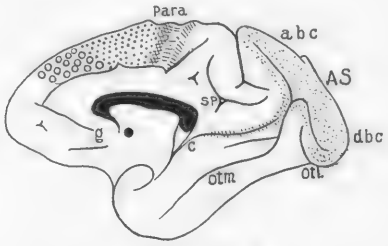


TEXT-FIG. 6.

TEXT-FIGS. 5 and 6. Extension of the area striata in the orang-utang, convex and mesial surfaces; after Brodmann.

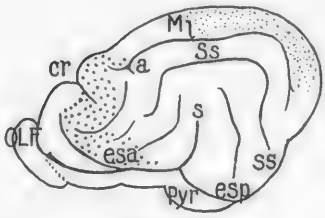


TEXT-FIG. 7.

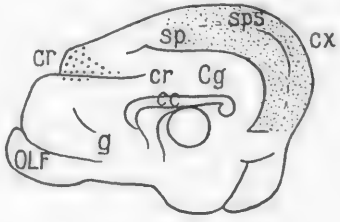


TEXT-FIG. 8.

TEXT-FIGS. 7 and 8. Extension of the area striata in the *cercopithecus,* convex and mesial surfaces; after Brodmann.



TEXT-FIG. 9.



TEXT-FIG. 10.

TEXT-FIGS. 9 and 10. Extension of the area striata in the occipital lobe of the cat, convex and mesial surfaces.

lamina granularis interna becomes narrower, the layer of pyramids becomes larger, and at the same time larger pyramidal cells appear in it.

The extension of the area striata shows, especially in the caudal and frontal ends, significant individual variations, which are confined to the mesial surface of the hemisphere. In the greatest number of cases it goes over on the convexity of the occipital lobe where it occupies the caudal pole.

In the anthropomorphous ape (text-figures 5 and 6 (orang-utang)) the area striata is extended over the mesial and convex surfaces of the occipital lobe approximately in equal parts.

In the *cercopithecus,* the long tailed monkey, the area striata occupies on the convexity the whole operculum between the sulcus similis and the fissure occipito temporalis lateralis. On the mesial surface the caudal pole is found around the ascending and descending branch of the fissure calcarina, while at the trunk of the fissure it soon disappears into the deep part of the fissure.

In carnivorous animals (text-figures 7 and 8) (cats and dogs) the area striata occupies principally the mesial and cerebellar surface of the occipital lobes. Their extension has been studied by Campbell, Brodmann, and Minkowski, whose results were the same (3, 4). The position of the area striata of the cat is marked by the caudal two-thirds of the gyrus marginalis in its mesial and lateral parts (text-figures 9 and 10).

The dog has a similar extension to that of the cat. The relation of this type of cortex to the act of vision was known by the earliest authors who had noticed after lesions in the neighborhood of the fissure calcarina lasting defects or scotoma in the field of vision.

Henchen and a number of other investigators have, by means of pathological lesions in the occipital lobes of man, marked out, as a clinical anatomical visual sphere, a zone whose destruction, in the minimum, is necessary to bring forth a maximum disturbance of vision. This zone consists in the cortex of the fissure calcarina, the neighboring lobes of the cuneus, and lobus lingualis; and therefore coincides with the extension of the area striata. The agreement about clinical and anatomical results that have been obtained has not remained without contradiction, as von Monakow explains

that he cannot accept, without further comment, Henchen's localization of the visual sphere in a relatively narrow part of the mesial surface of the occipital lobe, because of general pathological and anatomical reasons. He thinks it more likely that in all cases that have been described till now of the so called cortical lesion in the sphere of the calcarina, the optic radiation would also be damaged, that an extension which remains confined on the calcarine cortex is impossible, and therefore in calcarina lesions the occipital field cortex, which is separated from the primary occipital center, has really a larger extension and corresponds with the diseased area of the regio calcarina. von Monakow includes also in the anatomical clinical visual sphere, besides the regio calcarina with its specific cortex type, the first to the third occipital convolutions, the whole cuneus, the lobus lingualis, and the gyrus descendens. Experimental physiology seems to confirm this opinion. Minkowski's experimental research in the dog indicates that the clinical, anatomical visual sphere is covered with the cytoarchitectonic sphere, as lasting visual trouble can be caused only by extirpation of the area striata. Its total extirpation probably produces a maximal visual disturbance that can be reached from the cortex, while extirpation on the convexity of the occipital lobes, according to Minkowski, has as a consequence no, or only a short or quickly passing restriction of the visual field if they remain confined to the cortex and do not affect the optic radiation.

A further question arises: Is the cytoarchitectonic visual sphere close to the pathological anatomical sphere? von Monakow means by this the ideal convolution district in the occipital lobes whose whole destruction is just sufficient to bring forth a complete secondary degeneration of the primary optical centers and specially of the external geniculate body.

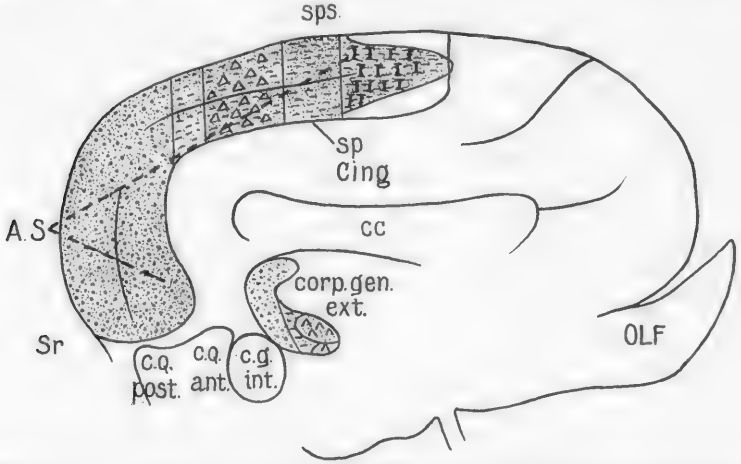
Here also human pathology can not give a precise answer. It is known, since the classical works of von Monakow, that the subcortical principal visual center of mammals, the corpus geniculatum externum, depends on the cortex, as it undergoes secondary degeneration through destruction of the occipital lobes. Until recently it was not possible to mark exactly on the cortex the district of representation of the corpus geniculatum externum. Only lately

has Minkowski succeeded in clearing up this point. He has experimental proof that the district of representation on the cortex of the cat by the corpus geniculatum externum is covered by the area striata; as only from this district can a secondary degeneration of the corpus geniculatum externum be produced by total extirpation of the area striata, the part most easily reached from the cortex, while the extirpation from the convexity of the occipital lobes outside the area striata produces in the corpus geniculatum externum a local degeneration, not a general volume reduction. If we consider the pathological anatomical visual sphere as the representative district of the corpus geniculatum externum, we may say that it is covered, in cats and dogs and probably in man, with the clinical anatomical and cytoarchitectonic visual sphere; that is, with the area striata.

A few words more about the projection of the corpus geniculatum externum and the area striata; that is, about the closer relation between certain parts of the area striata and the corpus geniculatum externum. It is known from human pathology that such a projection may exist; because through the destruction of the dorsal part of the visual cortex, that is, of the dorsal lip of the fissure calcarina and of the cuneus, the dorsal part of visual radiation, and, further, the dorsal part of the lateral fibers of the corpus geniculatum externum, the frontal mesial part degenerates secondarily; on the other hand, a secondary degeneration of the ventral part of the optic radiation and the caudal lateral part of the corpus geniculatum externum takes place after a lesion of the ventral half of the occipital lobes; that is, of the ventral lip of the calcarina and the lobus lingualis.

Minkowski has succeeded in showing the exact projection of the corpus geniculatum externum over the cortex and specially over the area striata of the cat. He has demonstrated that the frontal and upper parts of the area striata are in connection with the frontal part of the corpus geniculatum externum and the back, and at the same time the under parts of the area striata, with the back parts of the corpus geniculatum externum, so that after a partial extirpation of the area striata only the corresponding part of the corpus geniculatum externum degenerates secondarily. This projection is so sharply marked that it can be proved by a small cortex extirpation of 0.5 of a centimeter in diameter out of the area striata; because

after such an extirpation, a distinctly well marked island-shaped district of the corpus geniculatum externum undergoes secondary degeneration (text-figure 11).



TEXT-FIG. 11. Scheme of projection of the corpus geniculatum externum over the area striata in the cat.

SUMMARY.

In the study of the human cortex it seems well to follow the same primary divisions for the entire cortex, while in certain areas some layers may assume certain modifications; *viz.*, in the area striata it seems clearer to retain the sixth layer ground type, of which the fourth is subdivided into three secondary layers. The visual cortex, on account of its relation to the act of seeing, is an especially interesting field for study. About the calcarine fissure one can readily see with the naked eye a stripe of white fibers lying in the gray substance, called stria of Gennari or of Vicq-d' Azyr. This layer is considered by Brodmann in the second subdivision of the fourth primary layer.

The two most characteristic types of the cortex are the giant pyramid type, called area gigantopyramidalis, and the calcarine type, called area striata. The higher types of mammals afford excellent material for comparison.

Minkowski's experimental study of the dog indicates that the

clinical, anatomical visual sphere is covered with the cytoarchitectonic sphere; for lasting visual trouble can be caused only by extirpation of the area striata.

The subcortical visual center in mammals undergoes secondary degeneration through destruction of the area striata, and therefore depends on the cortex. Also certain parts of the corpus geniculatum externum have an exact projection over certain areas of the cortex.

ABBREVIATIONS USED IN THE TEXT-FIGURES.

cc,	corpus callosum.	abc,	ascending calcarina.
c,	calcarina.	dbc,	descending calcarina.
po,	parieto occipital.	g,	sulcus genualis.
ip,	intraparietal.	M,	gyrus marginalis.
os,	opercularis superior.	Ss,	gyrus supra Sylvius.
t_1,	first temporal.	Pyr,	lobus pyramidalis.
t_2,	second temporal.	Cg,	cingulum.
AS,	area striata.	Olf,	olfactory.
coll,	collateral.	!,	sulcus lateralis.
sp,	sulcus splenialis.	ss,	sulcus supra Sylvius.
s,	sulcus Sylvius.	esp,	sulcus ecto Sylvius posterior.
oi,	opercularis inferior.	esa,	sulcus ecto Sylvius anterior.
d,	descendens.	a,	sulcus ansatus.
si,	sulcus simialis.	cr,	sulcus cruciatus.
otl,	sulcus occipito temporalis.	sps,	sulcus supra splenialis.
cm,	calloso marginalis.	sr,	sulcus recurrens.
otm,	occipito temporalis medianus.	cx,	calcarine cortex.
otl,	occipito temporalis lateralis.	o,	operculum.
corp. gen. ext.,	external geniculate body.	Ld,	descendens lateralis.
		Para,	regio paracentralis.
c. g. int.,	internal geniculate body.	Cing,	cingulum.
c. q. ant.,	anterior corpora quadrigemina.		
c. q. post.,	posterior corpora quadrigemina.		

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A STUDY OF THE PATHOGENIC PROPERTIES OF BACILLUS PROTEUS.*

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Plates 65 to 67.

It is probable that no microörganism is encountered more frequently by those engaged in routine bacteriological work than some member of the *proteus* family, and yet there are few that at present attract less attention than these common bacteria. In fact, our standard text-books on bacteriology and pathology devote little or no space to these organisms.

The term *proteus* means a variety of form. Undoubtedly, many organisms have been classified as *proteus* bacilli which really do not belong to this class. Today the term *proteus* embraces such a large group of bacteria as to have no well defined limits.

GENERAL CHARACTERS OF PROTEUS BACILLI.

Hauser (1) described three types of *proteus* bacilli, classified according to their action upon gelatin. *Bacillus proteus vulgaris* is a rapid liquefier, liquefaction appearing in cultures from six to eight hours old. *Proteus mirabilis* liquefies more slowly. Both of these types, according to Hauser, form zoöglea in gelatin cultures. *Proteus zenkeri* neither liquefies gelatin nor forms zoöglea in this medium. These organisms are aerobic bacteria, growing readily on all culture media.

It is not the purpose of the present study to attempt to clarify the somewhat confused classification of the *proteus* group of organisms. We shall limit ourselves to a brief description of those with which we have experimented.

The *proteus* bacilli are Gram-negative, actively motile organisms, whose morphological characters resemble those of the colon typhoid

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group. The Gram-positive, spore-forming organisms described by some writers, we believe, should not be classified with the *proteus* family.

The strains with which we have experimented showed beginning liquefaction within twenty-four hours. Liquefaction has never been observed earlier than twelve hours. Milk was curdled readily, and the coagulum redissolved in about ninety-six hours. Loeffler's blood serum was usually liquefied. When cultivated on the various sugar media, the *proteus* resembles the paratyphoid bacilli. Dextrose and maltose are fermented with acid and gas formation. Saccharose is unaffected by most strains. Lactose, as a rule, is not fermented.

Jordan (2) found that the ratio of hydrogen to carbon dioxide formed in dextrose media is characteristic of the *proteus*. According to this author the amount of hydrogen formed always exceeds that of carbon dioxide; and this fact may be used as an aid in identification. Indol is formed in very perceptible proportions.

Widely divergent opinions prevail relative to the pathogenic properties of the *proteus* organisms. Many authors regard them as obligatory saprophytes. There is ample evidence in the literature, however, to show that they may at times become pathogenic.

In reviewing the literature in an attempt to determine precisely with what diseased conditions the *proteus* has been observed, one is handicapped by the imperfect descriptions of the organisms termed *proteus*. In fact, in some cases only the morphology of the germ is discussed, and it appears that a few of the earlier writers have felt justified in classing any pleomorphic bacillus as a *proteus*.

Foà and Bonome (3) recovered an organism which they believed to be the *proteus* from a case of volvulus in the year 1889. About a year later Krogius (4) reported a case of cystitis caused by the *proteus*. In the same year Schnitzler (5) reported a series of nine cases of cystitis due to the same organism. Jaeger (6) studied a series of ten cases of Weil's disease, some of which came to autopsy, in infections which occurred in the garrison at Ulm. He concluded that Weil's disease was a specific infectious disease due to *Bacillus proteus*.

In this country a *proteus* infection was described as early as 1892 by Flexner (7), who isolated *proteus vulgaris* from a case of peritonitis which had terminated fatally. Since then it has been found in a multitude of diseased conditions, such as pyelonephritis, prostatitis, enteritis, abscesses of various localities, caries of the bone, pleuritis, meningitis, arthritis, osteomyelitis, and gangrene. Not only man but some of the domestic and even the cold-blooded animals are subject to *proteus* infections. Jensen (8) has repeatedly found the *proteus* to

be the cause of diarrhea of calves. Jaeger described an epidemic of fowls in which the *proteus* likewise was found to be the infectious agent. He isolated this organism from chickens, geese, and ducks which had succumbed to the infection. In the year 1897 Wyss (9) studied an epidemic among the fish in Lake Zurich, which was, he believed, caused by *proteus vulgaris.* He recovered the organism from thirteen carcasses.

SOURCES OF PATHOGENIC PROTEUS STRAINS.

The first *proteus* strain with which we experimented was isolated from a laparotomy wound in a case which subsequently terminated fatally. The *proteus* was repeatedly isolated from this patient in pure culture. The second strain was isolated from a severe eye infection following a cataract operation. A third strain was isolated from an infection of the finger, believed to have been contracted at an autopsy. A fourth was isolated from the heart's blood of a case which had died from peritonitis following a gunshot wound of the intestines. The fifth strain was from a case of gangrene of the lung.

These organisms all proved to be pathogenic for rabbits. In our preliminary report (10) we have called attention briefly to some of the histological changes produced by *Bacillus proteus.*

Below we give the details of the experiments conducted during the past three years.

DESCRIPTION OF EXPERIMENTS.

Strain A.—Several strains of *proteus* bacilli have been used. Strain A was obtained from a suppurating laparotomy wound through which a tuberculous kidney had been removed. The following twenty-six experiments were made with this strain.

Experiment 1.[1]—Rabbit. Apr. 29, 1912. Inoculated intraperitoneally with one-fourth of a twenty-four hour slant agar culture obtained directly from the laparotomy wound. May 11. Death. Loss of weight, 25 per cent. At autopsy numerous soft, whitish nodules were found on the mesentery; also a few on the diaphragm and posterior abdominal wall. The lesions were in the peritoneum and subjacent tissue.

Microscopic Examination.—The nodules are composed mainly of a mass of polymorphonuclear leucocytes surrounded by a well developed wall of young connective tissue. There is necrosis in the central part of some of the abscesses. *Bacillus proteus* was recovered in pure culture from the lesions.

[1] These serial numbers are used for convenient reference. They do not always indicate the exact order in which the experiments were performed.

Experiment 2.—Rabbit. May 13, 1912. Inoculated intraperitoneally with a loopful of a twenty-four hour agar culture from experiment 1. May 30. Killed. Loss of weight, 11 per cent. At autopsy a number of small, whitish nodules were found on the omentum, mesentery, and liver.

Microscopic Examination.—The nodules are composed mainly of large connective tissue cells of the so called epithelioid type (figures 1 to 4). This is a typical proliferative inflammation. In histological structure it resembles an epithelioid cell tubercle. It will be described more fully below. One of the chief objects of our subsequent experiments with *B. proteus* was the study of this peculiar proliferative lesion.

Experiment 3.—Rabbit. May 20, 1912. Inoculated intravenously with 1 c.c. of a forty-eight hour broth culture from experiment 1. This broth culture was taken from an agar culture seven days old. May 27. Killed. Loss of weight, 10 per cent. At autopsy a large number of whitish nodules, some attaining a diameter of 5 mm., were seen in the liver. The lesions are scattered through the parenchyma of the organ. Similar nodules are found in both kidneys. The right lung contains large abscesses which have destroyed a considerable part of the organ.

Microscopic Examination.—The lesions are ordinary abscesses, consisting mainly of polymorphonuclear leucocytes. There is very little tendency toward encapsulation of the abscesses. *B. proteus* was obtained in pure culture from the lesions. This was the only instance in which we succeeded in producing macroscopic lesions by intravenous inoculation.

Experiment 4.—Rabbit. May 20, 1912. Inoculated intraperitoneally with 1 c.c. of the same culture used in experiment 3. May 25. Killed. Loss of weight, 19 per cent. At autopsy the omentum, mesentery, and the abdominal surface of the diaphragm were studded with small whitish nodules.

Microscopic Examination.—The nodules are composed of large connective tissue cells of epithelioid type, among which are a variable number of large and small mononuclear leucocytes. In some nodules the leucocytes predominate. No polymorphonuclear leucocytes are present. The lesions are, therefore, partly of proliferative and partly of exudative character. *B. proteus* was recovered in pure culture.

It will be noted that the original culture produced abscesses only (experiment 1). Cultures from experiment 1, injected intraperitoneally, produced proliferative lesions in experiment 2, and mixed exudative and proliferative lesions in experiment 4. When injected intravenously, the culture from experiment 1 produced abscesses (experiment 3).

Experiment 5.—Rabbit. June 7, 1912. Inoculated intraperitoneally with one-half of a twenty-four hour agar culture, taken from an agar culture which had stood in the laboratory since Apr. 16. No symptoms of infection appeared. June 13. Killed. Increase in weight, 1 per cent. No lesions were found at autopsy.

Experiment 6.—Rat. June 11, 1912. Inoculated intraperitoneally with 3 c.c.

of an eighteen hour broth culture, taken from the agar culture which had stood in the laboratory since Apr. 16. No symptoms of infection developed. June 17. Killed. Loss of weight, 10 per cent. No lesions were found at autopsy.

Experiment 7.—Rabbit. June 13, 1912. Inoculated intravenously with the same culture used in experiment 5. June 19. Killed. Loss of weight, 2 per cent. No lesions were found at autopsy.

These three experiments (5, 6, and 7) show that strain A had lost its virulence entirely by being kept in the laboratory for about two months.

Experiment 8.—Rat. June 11, 1912. 3 c.c. of an eighteen hour broth culture from experiment 4 were injected into the peritoneal cavity. Symptoms of infection developed and gradually became more severe. June 17. Animal moribund. Killed. Loss of weight, 29 per cent. At autopsy yellowish white nodules were found on the omentum, diaphragm, and liver.

Microscopic Examination.—Not made, but *B. proteus* was recovered in pure culture from the lesions.

Experiment 9.—Guinea pig. June 13, 1912. Inoculated intraperitoneally with 2 c.c. of a twenty-four hour broth culture from experiment 4, taken May 25. June 19. Killed. Loss of weight, 17 per cent. Large whitish nodules were found in the omentum at autopsy.

Microscopic Examination.—The nodules consist of epithelioid cells among which are a considerable number of polymorphonuclear leucocytes. The lesions represent a mixture of the exudative and the proliferative types of inflammatory reaction. They resemble the lesions found in experiment 4, except that the leucocytes in this instance are of the polymorphonuclear type.

Experiment 10.—Rat. June 19, 1912. 4 c.c. of a twenty-four hour broth culture from experiment 8 were given intraperitoneally. Death occurred within a few hours. The organism was recovered from the peritoneal cavity.

Experiment 11.—Rat. June 19, 1912. 3 c.c. of the same culture used in experiment 10 were injected. Death occurred the following night. The organism was recovered from the peritoneal cavity.

Experiment 12.—Rat. June 19, 1912. Intraperitoneal injection with 2 c.c. of the same culture used in experiment 10. Animal was sick the following day, but recovered. Increase in weight, 15 per cent. June 24. Killed. No lesions were found at autopsy.

Experiment 13.—Rat. June 19, 1912. 1 c.c. of the same culture used in experiment 10 was injected. No symptoms developed. Increase in weight, 5 per cent. June 24. Killed. No lesions found.

Experiment 14.—Rats. June 20, 1912. Three rats were each inoculated with 1.5 c.c. of a broth culture from experiment 8. All died the following night. The organism was recovered from the peritoneal cavity.

The preceding five experiments show that a *proteus* strain which had recently produced lesions in rats may lose this property entirely, although it may still be toxic when given in large doses.

Experiment 15.—Rats. June 21, 1912. Three rats were each inoculated intraperitoneally with 1 c.c. of a seven hour broth culture from experiment 14. All three were found dead the next morning. The organism was recovered from the peritoneal cavity.

Experiment 16.—Rats. June 22, 1912. Four rats were each injected intraperitoneally with 0.25 c.c. of a twenty-four hour broth culture from experiment 14. The first three died the following night. The organism was recovered from the peritoneal cavity. The fourth was sick the next day but recovered. No lesions were found when it was killed nine days later.

Experiments 15 and 16 show that the toxicity of strain A for rats had been markedly increased by a series of animal passages.

Experiment 17.—Rats. June 24, 1912. Two rats were injected intraperitoneally with 1 c.c. of a washed broth culture of *proteus* from experiment 16. No symptoms of infection appeared. Both were killed seven days later. No lesions were found at autopsy.

It appears from this experiment that the toxic properties of this *proteus* strain bear no relation to its ability to produce lesions. This is the same strain that produced lesions in experiment 8. By a series of animal passages the toxicity became markedly exalted, but, when the toxins were removed, the organism was harmless.

Experiment 18.—Rabbit. Aug. 2, 1912. Injected intravenously with one-fourth of an agar culture from experiment 9, which had stood in the laboratory since June 19. Aug. 8. Animal was in good condition. Aug. 22. Death from an intercurrent infection. No *proteus* lesions were found at autopsy.

Experiment 19.—Rabbit. Aug. 16, 1912. Inoculated intraperitoneally with one-half of a twenty-four hour culture of the same organism used in experiment 18. The animal gained in weight for several days. Death from pneumonia. Nov. 3. No *proteus* lesions were found at autopsy.

Experiments 18 and 19 demonstrate that strain A had lost its virulence entirely. No further experiments were made until April, 1913. The *proteus* cultures were kept in the laboratory during the intervening period.

Experiment 20.—Rabbits. One of the old cultures from strain A, which had long since lost its virulence, was injected into the anterior chamber of the eye of a rabbit (experiment 20 a). Twenty-four hours later it was recovered and this culture was injected into the peritoneal cavity of another rabbit (experiment 20 b), Apr. 26, 1913. The latter died the next day. *B. proteus* was recovered in pure culture from the peritoneal cavity and heart's blood.

Experiment 21.—Rabbit. Apr. 27, 1913. Injected intravenously with the same culture that was used intraperitoneally in experiment 20 b. No symptoms of infection developed.

Experiment 22.—Rabbit. Apr. 28, 1913. Intraperitoneal injection with one-

half of an agar culture from experiment 20 b. May 3. Animal was moribund. Killed. Loss of weight, 35 per cent. The omentum, both surfaces of the diaphragm, and the parietal pericardium were studded with soft whitish nodules. There were also a few nodules on the intestines. Emaciation was extreme.

Microscopic Examination.—The nodules are composed mainly of polymorphonuclear leucocytes. The central portions are necrotic. *B. proteus* was recovered in pure culture.

Experiment 23.—Rabbit. Apr. 28, 1913. Injected intraperitoneally with one-half of an agar culture from experiment 20 b. The animal lost weight for a few days, but began to regain it. May 6. Killed. Large, yellowish white nodules were found in the omentum, mesocolon, and parietal peritoneum.

Microscopic Examination.—The lesions consist of masses of polymorphonuclear leucocytes surrounded by a zone of newly formed connective tissue. They are healing abscesses. *B. proteus* was recovered in pure culture.

Experiment 24.—Rabbits. May 5, 1913. Two rabbits were each injected intraperitoneally with one loop of an agar culture from experiment 22. No symptoms of infection developed.

Experiment 25.—Rabbit. May 7, 1913. Inoculated subcutaneously with one-half of an agar culture from experiment 23. May 9. Death from an intercurrent infection. A small abscess was found at the site of the injection, from which *B. proteus* was recovered in pure culture. Heart's blood negative.

Experiment 26.—Rabbit. May 7, 1913. Inoculated subcutaneously with an old culture of strain A which had been made virulent by passage through the anterior chamber of the eye of a rabbit, as in experiment 20 a. May 15. Killed. Loss of weight, 41 per cent. A large subcutaneous abscess was found at the site of the inoculation, from which *B. proteus* was recovered in pure culture.

Experiments 20 to 26 show that an avirulent *proteus* strain can be made virulent by inoculation into the anterior chamber of the eye. It is also seen that strain A does not now produce proliferative lesions.

Strain B.—This strain was isolated from an eye infection, following a cataract operation which necessitated removal of the eyeball.

Experiment 27.—Rabbit. May 28, 1912. Injected intraperitoneally with 1 c.c. of a twenty-four hour broth culture of this organism. Death occurred on June 4. Loss of weight, 16 per cent. Autopsy revealed several rather large abscesses in the omentum, from which *B. proteus* was recovered in pure culture.

Microscopic Examination.—The nodules were found to be typical abscesses.

Strain C.—Strain C was obtained from an infection of a finger, which was contracted while assisting at an autopsy.

Experiment 28.—Rabbit. July 29, 1913. Injected intraperitoneally with this culture. No symptoms of infection developed. Aug. 11. Laparotomy revealed a large number of peritoneal lesions. There had been only a slight loss of weight. Aug. 15. Killed. At autopsy a number of small whitish nodules were found on the omentum and colon.

Microscopic Examination.—The nodules showed a proliferative lesion similar to that described in experiment 2.

Experiment 29.—Rabbit. Aug. 18, 1913. Injected intraperitoneally with one-half of a five hour agar culture from experiment 28. Death occurred the following night.

Experiment 30.—Rabbit. Aug. 19, 1913. Injected intraperitoneally with a culture from experiment 28. Animal became ill and lost nearly 200 gm. in weight, but gradually recovered.

Experiment 31.—Rabbit. Sept. 4, 1913. Injected intraperitoneally with a culture from experiment 28. No symptoms of infection developed.

Experiment 32.—Rabbit. Sept. 26, 1913. Injected subcutaneously and intraperitoneally with a culture from experiment 28. No symptoms of infection appeared.

Experiment 33.—Rabbit. Sept. 26, 1913. Injected intraperitoneally with a culture from experiment 28. Sept. 30. Animal dying of an intercurrent disease. Killed. No lesions were found in the peritoneal cavity.

Experiment 28 shows that strain C had the power to produce lesions in rabbits when it was first recovered from human tissue. Experiments 31 to 33 show that this property rapidly disappeared when the organisms were grown in the laboratory.

Strain D.—Strain D was obtained from the heart's blood in a human autopsy. The main autopsy findings were a gunshot wound through the abdomen, producing multiple perforations of the intestines, general peritonitis, and lobar pneumonia. *Bacillus proteus* was also isolated from the peritoneal cavity.

Experiment 34.—Rabbit. Sept. 23, 1913. Injected intraperitoneally with one-half of a twenty-four hour agar culture of strain D. Sept. 24. Died. The peritoneal cavity contained a considerable amount of thin blood-stained fluid. *B. proteus* was recovered from this fluid. The plates contained an occasional staphylococcus colony.

Experiment 35.—Rabbit. Sept. 24, 1913. Injected intraperitoneally with a small dose of the culture used in experiment 34. Sept. 29. Killed. No lesions were found at autopsy.

Experiment 36.—Rabbit. Sept. 24, 1913. Inoculated subcutaneously with one-half of a five hour agar culture from experiment 34. Death occurred during the night.

Experiment 37.—Rabbit. Oct. 8, 1913. Injected subcutaneously with aggressin from a culture of strain D. Two hours later the rabbit was inoculated subcutaneously with a loopful of a culture of the same strain. Symptoms of severe infection appeared. A large subcutaneous abscess developed at the site of the inoculation. Oct. 14. The abdominal cavity was accidentally opened while exploring the abscess. Oct. 16. Death occurred from peritonitis. Loss of weight, 21 per cent. *B. proteus* was recovered in pure culture from the peritoneum as well as from the abscess.

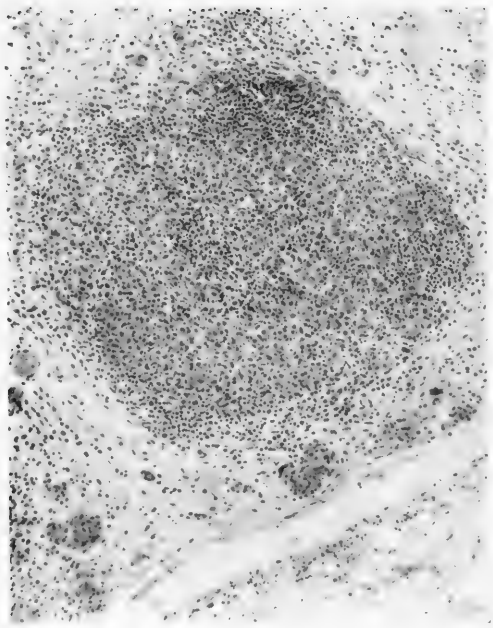

FIG. 1.

(Larson and Bell: Properties of *Bacillus proteus.*)

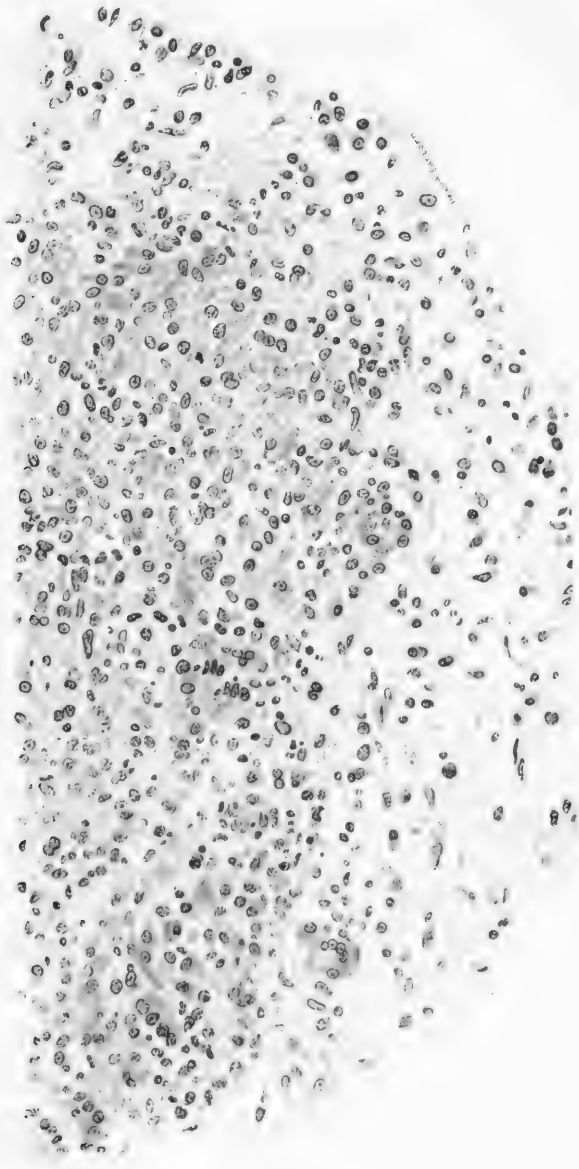

FIG. 2.

(Larson and Bell: Properties of *Bacillus proteus.*)

FIG. 3.

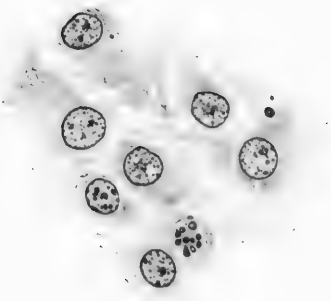

FIG. 4.

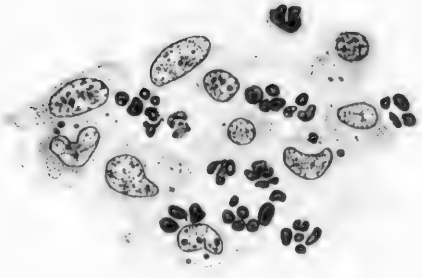

FIG. 5.

(Larson and Bell: Properties of *Bacillus proteus.*)

Experiment 38.—Rabbit. Oct. 8, 1913. Injected subcutaneously with aggressin from a culture of strain D. One hour later inoculated intraperitoneally with a loopful of a culture of the same strain. A large subcutaneous abscess developed at the site of the inoculation. Loss of weight in eight days, 22 per cent. After about four weeks the abscess began to disappear. Dec. 17. Death from an intercurrent disease. The abscess had disappeared entirely by this time. There were no *proteus* lesions present.

Experiment 39.—Rabbit. Oct. 15, 1913. Inoculated intraperitoneally with one-half of a twenty-four hour agar culture taken from experiment 37 at operation. Oct. 24. Died. Loss of weight, 30 per cent. Autopsy revealed a considerable number of whitish nodules, 1 mm. to 3 mm. in diameter, on the mesentery, omentum, diaphragm, and posterior abdominal wall.

Microscopic Examination.—Most of the nodules are ordinary abscesses; but a few show the proliferative type of lesion.

Strain E.—Strain E was obtained from a gangrenous human lung.

Experiment 40.—Rabbit. Dec. 3, 1913. Inoculated intraperitoneally with a twenty-four hour culture of this organism. Dec. 8. Died. At autopsy a large number of characteristic nodules were found on the peritoneal surfaces. Microscopically, some of the nodules were found to be ordinary abscesses. Other nodules show proliferative inflammation. *B. proteus* was recovered from the lesions.

Experiment 41.—Rabbit. Dec. 10, 1913. Inoculated intraperitoneally with one-half of an agar culture from experiment 40. Dec. 18. Killed. Loss of weight, 13 per cent. Numerous soft, whitish nodules were found on the peritoneal surfaces.

Microscopic Examination.—Some of the nodules are ordinary abscesses surrounded by a well developed wall of fibroblasts. Other nodules show mainly the proliferative lesion. There are masses of large connective tissue cells of the epithelioid type among which a considerable number of polymorphonuclear leucocytes are interspersed (figure 5).

Experiment 42.—Rabbit. Dec. 19, 1913. Inoculated intravenously with one-half of an agar culture from experiment 41. Died the next day. *Proteus* recovered from the heart's blood.

Experiment 43.—Rabbit. Dec. 23, 1913. Inoculated intraperitoneally with one-half of an agar culture from experiment 42. Dec. 30. Laparotomy showed no lesions in the peritoneal cavity.

Experiment 44.—Rabbit. Dec. 25, 1913. Injected intravenously with one-half of an agar culture from experiment 42. No symptoms of infection developed.

The proliferative lesion appeared with strain E in experiment 40, and again in experiment 41. It was not followed further, since the strain suddenly lost its virulence.

DISCUSSION.

From the above experiments it will be seen that some strains of *Bacillus proteus* obtained from human lesions are definitely patho-

genic for rabbits, rats, and guinea pigs, producing either typical abscesses or a granulomatous type of lesion. Not all strains of the *proteus* are pathogenic, however. During the past three years we have experimented with twenty other strains which were obtained from various sources, such as decaying organic material and routine autopsy blood cultures where they were probably merely postmortem invaders. In one case a non-pathogenic strain was isolated from an amebic abscess of the liver. None of these proved to be pathogenic for laboratory animals. Rabbits injected either intravenously or intraperitoneally with one to two cubic centimeters of broth cultures of such strains suffered little or no inconvenience. Where excessive doses were given the animals died with symptoms of acute toxemia.

The five strains of *proteus* which were pathogenic for laboratory animals were obtained from human lesions from which, with one exception (strain E), no other organisms were recovered. It is, therefore, probable that these organisms were responsible for the lesions in which they were found. Only in two cases (strains C and E) was it possible to examine microscopically the tissues from which the organisms were obtained. Sections from the infected finger (strain C) showed atypical tubercles, but repeated examinations failed to reveal acid-fast bacilli. However, the possibility of a tuberculous infection in this case cannot be excluded. Strain E was obtained from a gangrenous lung in association with pneumococci. No cellular structure was visible in the area from which the culture was taken. Outside the gangrenous area the lung showed typical lobar pneumonia.

From our work covering a study of twenty-five different *proteus* strains we have come to regard a positive rabbit inoculation as strong evidence that the strain is pathogenic for man. It must be borne in mind that a positive inoculation does not necessarily cause the death of the animal, since some animals may recover from rather severe infections. The method of inoculation is very important. The intraperitoneal inoculation is the method of choice, since intraperitoneal infections are more severe than subcutaneous infections. Inoculations made intravenously rarely result in the production of the characteristic lesions.

The toxic properties of the *proteus* bacilli must not be confused with their power of producing the characteristic inflammatory reactions described in our experiments; in fact, these two characters bear no relation to each other. As indicated in experiments 10 to 16, the toxicity of the organism may become most markedly exalted, while its power to produce the lesions is entirely lost.

Proteus cultures lose their virulence rapidly when kept on laboratory media. The original culture of strain A, which was at first very virulent, became non-pathogenic in about two months (experiments 5, 6, and 7). Another culture of this strain lost its virulence in six weeks (experiments 18 and 19). Strain C became avirulent in three weeks (experiment 31); and strain E in less than one week (experiments 43 and 44). This rapid loss of virulence may explain the fact that *Bacillus proteus* is seldom regarded by bacteriologists as an important pathogenic organism.

A non-pathogenic *proteus* may be made pathogenic by inoculation into the anterior chamber of the eye. In this situation the organisms grow rapidly, there being neither antibodies nor complement present. If recovered from the anterior chamber after twenty-four hours, the culture will usually be found to be pathogenic (experiments 20, 22, and 23). Non-pathogenic strains of streptococci may also become very virulent for rabbits when grown in the anterior chamber, as just described.

A non-pathogenic *proteus* may be enabled to produce lesions by the use of aggressins. The aggressin is injected one or two hours before the animal is inoculated with the bacteria (experiments 37 and 38).

Strains of *proteus,* made pathogenic by either of the above described procedures, produce lesions similar to those produced by strains that were pathogenic when obtained from human lesions.

SYMPTOMS OF PROTEUS INFECTION IN LABORATORY ANIMALS.

The clinical symptoms of experimental *proteus* infection in animals present marked variations, depending mainly upon the virulence of the bacteria. In the mildest cases the animals may not show any signs of infection. In one case (experiment 28), which presented no clinical symptoms, laparotomy revealed rather extensive peritoneal lesions. Mild cases show a temporary loss of weight, weakness, and

decreased appetite, after which they gradually recover. Occasionally an animal will recover from a very severe infection (experiment 38).

Several of the rabbits that were killed might otherwise have recovered from the *proteus* infection.

In very severe *proteus* infections the animals become extremely emaciated. In one case a loss of weight of 41 per cent. was recorded. Losses of 20 to 30 per cent. are common. Emaciation is a striking feature of severe *proteus* infections. The animals refuse to eat and become progressively weaker. Death usually results in about one week. Deaths from toxemia, without the production of lesions, usually occur within the first twenty-four hours.

GROSS AND MICROSCOPIC PATHOLOGY.

The characteristic lesions resulting from intraperitoneal inoculation are fairly firm, whitish nodules, one to five millimeters in diameter, sometimes larger. They may occur on any part of the peritoneal surface. They were most frequently found in the omentum in our experiments, apparently because the injecting needle came into contact with this structure. The number and size of the lesions usually corresponded to the clinical severity of the infection. In one case the infection passed through the diaphragm and involved the lungs and pleuræ. In no other instance did the lesions resulting from an intraperitoneal inoculation extend beyond the peritoneal cavity.

The large nodules with softened centers always show the exudative type of lesion histologically; but, as a rule, the exudative and proliferative lesions cannot be distinguished by macroscopic examination. When subcutaneous inoculation is successful it usually results in the formation of an abscess of varying size. Death may result from a subcutaneous lesion; but an intraperitoneal infection advances more rapidly and is more apt to terminate fatally.

By intravenous inoculation we succeeded in producing the characteristic lesions in only one case (experiment 3). In this instance there were abscesses in the liver, both kidneys, and the right lung. *Bacillus proteus* disappears rapidly from the blood stream. After intravenous inoculation it was recovered only in those cases that died within the first twenty-four hours. It was never recovered from

the heart's blood after subcutaneous inoculations, and only once after intraperitoneal injection.

As regards histological structure the *proteus* lesions show an exudative or a proliferative type of inflammation, or a mixture of these two forms of tissue reaction. The exudative lesions occur more frequently. They are simple abscesses composed mainly of polymorphonuclear leucocytes. If the infection is not too severe, the abscesses become walled off by a zone of newly formed connective tissue. In the one case in which intravenous inoculation was successful, typical abscesses were formed. The subcutaneous lesions in every case were of the exudative type. The exudative lesions have no special histological interest since they are ordinary abscesses.

The proliferative lesions, however, demand particular attention. Grossly, the nodules are usually smaller and firmer than the abscesses, but it is often not possible to distinguish them from the exudative lesions by gross examination. Figure 1 represents a small nodule of the proliferative type under low magnification. It consists mainly of large cells resembling the so called epithelioid cells of a tubercle. This lesion may indeed be grouped with the granulomata. Masses of small lymphocytes are seen around the periphery of the nodule and in certain places within it. Below the large nodule are seen several smaller ones which consist of a few epithelioid cells. In a section through one of the macroscopic omental nodules of this case, one sees several masses resembling in structure the one shown in figure 1, but some of them are many times larger.

A higher magnification of a part of a proliferative lesion is shown in figure 2. There is a gradual transition from the dense part of the nodule to the adjacent fibrous connective tissue. The cytoplasm of some of the epithelioid cells fuses with that of adjacent cells. Blood vessels are not present in the denser parts of the lesion.

Figures 3 and 4 represent small areas from figure 2 under very high magnification. Multinucleated cells are shown in figure 3. The epithelioid cells are frequently found fused, in this way forming giant-cells, but no giant-cells of the Langhans type have ever been observed in any case. Figure 4 represents an area in which the cells are all distinct. In both figures delicate connective tissue fibrillæ are shown running between the cells and apparently con-

nected to their cytoplasm. By means of Mallory's phosphotungstic hematoxylin stain, it is easy to see that these fibrillæ are present everywhere among the epithelioid cells.

The smallest recognizable nodules, such as are shown in the lower part of figure 1, consist of a few epithelioid cells. The delicate fibrillæ are always present except where the cytoplasm of the cells is fused. Careful examination of the peripheral parts of the nodules (figure 2) will show that there is a gradual transition between the epithelioid cells and the adjacent fixed connective tissue cells. There is no evidence that the epithelioid cells are derived from the endothelium of blood or lymph vessels. All the evidence available indicates that they develop from the fixed cells of the connective tissue. The black masses shown in the cytoplasm of the cells in figures 3 and 4 are fragments of the nuclei of leucocytes. In some proliferative lesions all the epithelioid cells are crowded with coarse, deeply stained nuclear fragments. These cells readily ingest the leucocytes that enter the lesion.

Frequently the nodules consist of epithelioid cells among which are numerous polymorphonuclear leucocytes. Such a lesion is shown in figure 5. This may be regarded as a mixture of the exudative and proliferative types of inflammation. As in figure 5, the leucocytes in these lesions usually show pronounced signs of degeneration. They evidently degenerate readily and are ingested by the epithelioid cells.

Sections appropriately stained[2] show enormous numbers of bacilli everywhere in the proliferative lesion. The bacilli all seem to be extracellular in position.

There seems to be no relation between the clinical severity of the case and the histological type of the lesion. Neither does the histology of the lesion depend upon the particular strain of *proteus* used, since all types of lesions were produced at different times with strain A. The virulence of the particular culture employed, though it may be of importance, is not the only factor, since both exudative and proliferative lesions were sometimes found close together in

[2] To stain the bacilli the tissues should be fixed in Zenker's fluid, sectioned in paraffin, and stained by Mallory's eosin-methylene blue method (overstaining with the methylene blue). This method was used by Mallory to show the Bordet-Gengou bacillus in tissue sections.

the same experiment (experiments 39, 40, and 41). We did not discover the factors that control the histology of the lesion.

SUMMARY.

Some strains of *Bacillus proteus* obtained from human lesions are pathogenic for rabbits, rats, and guinea pigs. There is good evidence that these strains are also pathogenic for man.

All cultures obtained from sources other than human infections were non-pathogenic for the laboratory animals.

A non-pathogenic culture may be made pathogenic by the use of aggressins or by inoculation into the anterior chamber of the eye.

Proteus cultures lose their virulence rapidly when grown on artificial media.

The lesions produced in animals are either simple abscesses, proliferative lesions, or a mixed exudative and proliferative lesion.

The proliferative lesions consist mainly of epithelioid cells apparently of connective tissue origin. No giant-cells of the Langhans type are present.

The histological type of the lesion does not depend upon the strain employed. Neither does it bear any relation to the clinical severity of the case.

The ability to produce the characteristic lesions has no necessary connection with the toxicity of the bacteria.

The *proteus* bacteria probably play a more important part in human pathology than is generally believed.

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EXPLANATION OF PLATES.

PLATE 65.

FIG. 1. Experiment 2. Proliferative lesion from the omentum. Several very small nests of epithelioid cells are shown in the lower part of the figure. Microphotograph. × 120.

PLATE 66.

FIG. 2. Experiment 2. Proliferative lesion. One-half of a nodule similar to the one shown in figure 1. × 350.

PLATE 67.

FIG. 3. Experiment 2. Small area from figure 2. Note the fusion of the epithelioid cells. Delicate connective tissue fibrillæ are shown. The black mass in the cytoplasm is a pycnotic nucleus. × 900.

FIG. 4. Experiment 2. Small area from figure 2. Connective tissue fibrillæ are prominent. A disintegrating leucocyte is shown in the cytoplasm of one of the cells. × 900.

FIG. 5. Experiment 41. Mixed exudative and proliferative lesion. The polymorphonuclear leucocytes show various stages of degeneration. × 900.

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